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A Fully Automatic Apparatus for Solid-liquid Phase Counter-current Distribution

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(*Received* 9 *February* 1953)

In recent years much attention has been attracted to the use of discontinuous counter-current extraction between a pair of immiscible liquid phases as a method of purification. Craig and his associates (for references see Craig & Craig, 1950) have suggested two forms of apparatus suitable for this purpose. Craig, Hausmann, Ahrens & Harfenist (1951) have described an all-glass unit in which the processes of equilibration, settling of the two phases and transfer of the upper phase to the next unit can all be carried out automatically. The complete apparatus has 220 of these units. Fresh solvent was fed into the first tube and fractions collected automatically from the last tube, so that the whole operation was analogous to chromatography.

Discontinuous counter-current distribution between liquid and solid phases has attracted less attention, although Craig, Golumbic, Mighton & Titus (1946) described the separation of chrysene and anthracene by solid-liquid phase counter-current distribution between alumina and *cyclo*hexane. The separation was carried out manually in glass tubes, the *cyclo*hexane phase being transferred by decantation to the next tube after each equilibration.

The present paper describes a fully automatic apparatus for carrying out the stages of equilibration, filtration and transfer of the filtrate to the next unit. The advantages of automatic operation, stressed by Craig & Craig (1950) for liquid-liquid systems are even more marked for solid-liquid systems where both equilibration and filtration stages may be slow.

Solid-liquid phase counter-current distribution is very similar to adsorption chromatography and at first sight may appear to offer little advantage over the latter. However, when equilibrium of a solute between solid and liquid phases is slow and it is desired to operate in exactly defined non-equilibrium conditions, the method offers certain advantages. Results obtained with batch experiments, especially when the adsorption isotherm shows marked curvature, can be readily transferred to cascade operation.

APPARATUS AND METHOD

The apparatus consists essentially of two parts, the glass units and the electrical and mechanical equipment necessary for the operation cycle.

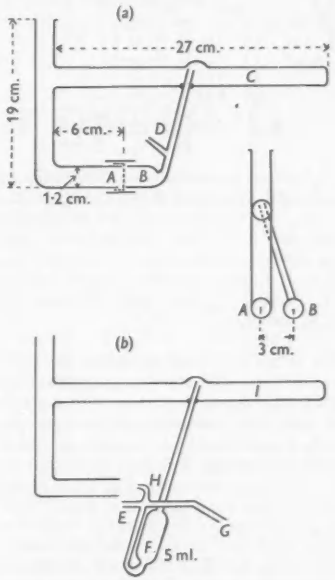

Fig. 1. Glass units for solid-liquid phase counter-current distribution. (*a*) Main units; (*b*) special metering unit.

The standard glass unit is shown in Fig. 1 (*a*). The dimensions shown are suitable for liquid-phase volumes of 5 ml. but the units can of course be scaled up or down. Part *A* of each unit is joined to part *B* of the next unit by a sleeve of plastic tubing. This sleeve also holds in position a disk of filter paper (preferably Whatman no. 54), placed between the two units. Equilibration takes place in tube *A* by oscillation through an angle of about 15° on either side of the horizontal position. At the end of the equilibration period, the unit is rotated through 90° in a clockwise direction. The liquid phase in *A* then filters through *B* into *C*, *D* is an air

vent. Rotation of the unit through 90° in an anticlockwise direction then transfers the liquid in *C* into the *A* portion of the next unit. Any desired number of units can be assembled into the train. Fig. 1 (*b*) shows the special first unit which delivers a constant volume of fresh liquid phase into tube O of the train. Fresh liquid phase enters continuously from a reservoir via tube *E* and fills the bulb *F* (5 ml. capacity). Excess liquid flows over into the drain tube *G*. Maintenance of a constant level is facilitated by the oscillating movement of the whole unit. During the filtration stage when the unit has been rotated through 90° in a clockwise direction, the contents of *F* are transferred to *I*, tube *H* acting as an air vent. During this stage, liquid phase entering through *E* passes directly out through *G* and does not enter the bulb *F*.

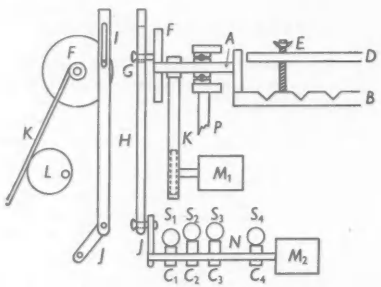

Fig. 2. Actuating mechanism for automatic operation. *A*, main shaft; *B*, notched supporting bar for glass units; C_1, C_2, C_3, C_4, actuating cams for switches; *D*, upper clamping bar; *E*, locating bolts and wingnuts; *F*, main driving wheel; *G*, driving pin on *F*; *H*, slotted driving bar; *I*, slot in *H*; *J*, driving crank; *K*, cam follower; *L*, cam; M_1, M_2, motors; *N*, timing shaft; *P*, main support; S_1, S_2, S_3, S_4, switches.

On return to the equilibration position the liquid in *I* is transferred to the equilibration tube *A* of the next unit. The liquid phases leaving the last tube of the train are collected in tubes held in a turntable-type fraction collector, an empty tube being brought into position once per cycle.

The mechanical design of the support for the units and the actuating mechanism is shown in Fig. 2. The support for the glass units is a duralumin bar *B*, 2 in. wide, 0·5 in. thick, of a length adequate to accommodate the required number of glass units. The bar is provided with equidistant machined notches to locate the units. The latter are fastened in place by the upper duralumin bar *D* and the bolts and wingnuts *E* spaced every five notches. The whole unit is carried on 0·5 in. shafts, *A*, at each end. These shafts run in self-aligning ballraces mounted on the main supports *P*. Shaft *A* carries the metal bar *K* and the wheel *F*. Bar *K* is kept in contact with cam *L* by a counter weight on *A*. *L* is driven by motor M_1. Operation of M_1 thus results in an angular oscillation of *A*, the magnitude of which is controlled by the size of *L* and the distance between the spindle of M_1 and *A*. An angular oscillation of about 30° has been used in the present apparatus. Shaft *A* also carries the wheel *F* with the pin *G* engaging in the slotted bar *H*. During oscillation of *A* by M_1 the pin *G* rides freely in the slot *I*. Rotation of crank *J* through 360° by motor M_2 causes rotation of shaft *A* through about 90° and back to the original position. This provides the filtration and transfer position for the glass units. The angular rotation of *A* can be varied by altering the throw of crank *J*. The spindle of motor M_2 carries, in addition to crank *J*, the three cams C_1, C_2, C_3, operating the three mercury switches S_1, S_2, S_3, which together with the timing clock, control the operating cycle.

The electrical circuit shown in Fig. 3 comprises essentially the two operating motors M_1, M_2, three mercury switches (230 v, 1 A rating) S_1, S_2, S_3 and the timing clock *T*. The latter is a Flexopulse signal timer (Eagle Signal Corporation, Moline, Ill., U.S.A.). It consists of a single-pole two-way switch, the duration of contact in each position being continuously and independently variable from 3 min. to 5 hr. Motor M_1 is provided with a rheostat, R_1, so that the

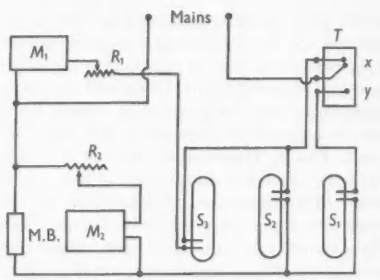

Fig. 3. Circuit arrangement of electrical components. M_1, M_2, motors; M.B., magnetic brake; R_1, R_2, rheostats; S_1, S_2, S_3, mercury in glass switches; *T*, Eagle signal timer.

speed of shaking can be varied. Motor M_2 has a magnetic brake in parallel with it to prevent overrunning of the cams C_1, C_2, C_3 when M_2 is switched off, and a rheostat, R_2.

The cycle of operating is as follows.

(1) The timer switch is in the *x* position, S_3 is closed so that motor M_1 is in operation for the equilibration period. S_1 is closed, S_2 is open, motor M_2 is thus switched off.

(2) At the end of the equilibration period determined by the timer *x* setting, the timer switch moves over to the *y* position. Motor M_1 is thus switched off. Since S_1 is closed, M_2 begins to rotate, opening S_3. Shaft *A* is rotated via *F*, *H* and *J* through approximately 90°, when the filtration position of the glass units is reached. At this position, cam C_1 opens S_1 switching off M_2. Simultaneously cam C_2 closes S_2.

(3) At the end of the filtration period determined by the timer *y* setting, the timer contact moves over to the *x* position. Since S_2 is now closed, motor M_2 begins to rotate and completes the remainder of its 360° rotation. S_2 is opened by C_2 at the end, thus switching off M_2. Shaft *A* is rotated through 90° in an opposite direction to its initial movement, thus completing the transfer stage of the glass units. When *A* is in its initial position, S_1 and S_3 are closed by C_1 and C_3. Motor M_1 is thus switched on, equilibration shaking begins and the cycle is completed.

Shaft *N* also carried a cam, C_4, operating a switch, S_4, which is closed momentarily once per cycle. This actuates the fraction collector, moving it on one tube. The fraction collector also serves as a counter for the number of cycles completed.

The mechanical and electrical mechanism can also be readily adapted for automatic operation of a liquid-liquid counter-current distribution apparatus. This only involves rotation of C_1 on N so that it opens S_1 rather earlier in the cycle, for example after A has rotated through about 30°. M_2 is then switched off and the glass units stay in the settling position, i.e. until the two liquid phases have separated, for the period determined by the timer y position. When the timer switches over to the x position, M_2 completes its 360° rotation as described above, A completes its clockwise 90° movement to the first transfer position and its anticlockwise return to the initial position. The upper phase in the Craig units is thus decanted into the upper tube and then passed on to the next unit.

SUMMARY

1. A glass counter-current apparatus suitable for working with solid-liquid systems is described. Provision is made for equilibrating a solute between the two phases, separation of the phases by filtration and transfer of the liquid phase to the next unit.

2. An electrically operated mechanism is described suitable for carrying out the operation cycle automatically.

3. Adaptation of the mechanism to operation of a liquid-liquid counter-current apparatus is also described.

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The Oxidation of Corticosteroids with Sodium Bismuthate

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(*Received* 27 *March* 1953)

Lead tetraacetate and periodic acid are the reagents commonly chosen for the selective fission of 1:2-glycols under mild conditions (cf. Jackson, 1944; Criegee, 1948; Johnson, 1951). Both reagents are also capable of cleaving 1:2-ketols, though for substances of this type their effectiveness can vary greatly with the molecular environment of the ketol grouping. The two oxidants differ in their action on α-hydroxy acids, which are cleaved by lead tetraacetate but remain unaffected by periodate. In general, when the effect of either reagent is expected to be the same, lead tetraacetate is taken for reactions in organic solvents, while periodate is preferred for oxidations in aqueous media and for analytical purposes.

Periodate oxidation has proved of great value in elucidating the structures of corticosteroids (cf. Reichstein & Shoppee, 1943), and forms the basis of the analytical determination of 'formaldehydogenic' steroids (Lowenstein, Corcoran & Page, 1946; Daughaday, Jaffe & Williams, 1948; Corcoran, Page & Dustan, 1950), 'acetaldehydogenic' steroids (Butler & Marrian, 1937, 1938; Cox, 1952) and corticosteroids which are converted by periodate to 17-ketosteroids (Talbot & Eitingon, 1944; Fieser, Fields & Lieberman, 1944). The application of lead tetraacetate in this field was less successful: Reichstein (1937) succeeded in converting 11β:17α:21-trihydroxypregn-4-ene-3:20-dione (V) to 11β-hydroxyandrost-4-ene-3:17-dione (XII) with this reagent, but observed that the reaction was sluggish and the yield poor.

A consideration of the mechanism of glycol fission with lead tetraacetate and with periodic acid led Heidt, Gladding & Purves (1945) to postulate certain physical properties required of any oxidant capable of cleaving glycols by the same mechanism. Two oxidants found to have such properties were tervalent silver and sodium bismuthate. The latter reagent, in particular, was shown to effect smooth cleavage of ethylene glycol: quantitative yields of formaldehyde were obtained by shaking a dilute aqueous solution of glycol (at pH $\leqslant$ 4·7) with a large excess of sodium bismuthate. The reaction was complete within 30 min. at room temperature. Rigby (1949, 1950) has independently investigated the action of sodium bismuthate on various simple 1:2-glycols, 1:2-ketols and α-hydroxy acids. The oxidations were performed, in general, with the theoretical amounts of reagent, the medium being either glacial acetic acid or an aqueous solution containing a slight excess of phosphoric acid. The author concluded that in its effect, selectivity and rate of reaction sodium bismuthate closely

resembles periodic acid and lead tetraacetate. In contradistinction to periodate, however, sodium bismuthate was found to cleave α-hydroxy acids.

It appeared to us that this last finding could be exploited for the characterization of the important group of corticosteroids bearing a dihydroxyacetone side chain (type (*d*), Table 1): whereas periodate degrades these only to the α-hydroxy acid stage, oxidation with bismuthate should convert them to 17-ketosteroids, which are relatively easy to characterize and to assay. Furthermore, the insolubility of sodium bismuthate promised to offer technical advantages over the use of periodate, e.g. in permitting the employment of a large excess of the reagent and simple separation of the surplus. Consequently, we have explored the usefulness of the bismuthate oxidation for the characterization of corticosteroids on both the preparative and the micro-analytical scale. Part of this work has been briefly reported in a preliminary communication (Brooks & Norymberski, 1952).

Seven compounds (I–VII, Table 1), representing the six typical corticosteroid side chains (*a*)–(*f*), have been chosen as model substances in this work. The 17:20-diol (VI) was prepared by catalytic hydrogenation of the 17:20-ketol (VII). The 20:21-diol (I) was prepared from 3β:21-dihydroxypregn-5-en-20-one 21-acetate (A; R′ = >CH_2, R″ = >CH.CO.CH_2OAc) by reduction with lithium aluminium hydride. This transformation has been previously effected by the Meerwein-Ponndorf method (Steiger & Reichstein, 1938; Ehrenstein, 1943; Fieser *et al.* 1944): from the difficultly separable mixture of 20-epimers thus obtained, Ehrenstein isolated one of the epimers, which had m.p. 222–229°, $[\alpha]_D - 54°$. From the lithium aluminium hydride reduction we obtained without difficulty a compound of the composition $C_{21}H_{34}O_3$, m.p. 221–224°, $[\alpha]_D - 56°$, identical with Ehrenstein's product, a specimen of which was kindly made available by Dr Ehrenstein.

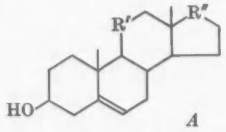
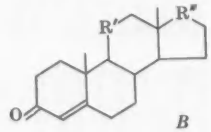
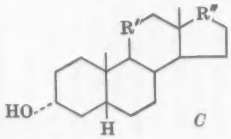
Acetylation of the mother liquors afforded a triacetate, m.p. 182–186°, $[\alpha]_D - 22°$, identical with that prepared from the pure 20:21-diol. The increment in molecular rotation on acetylation (Δ_1) is +81°. Since no vicinal action has been observed between the hydroxyl groups at $C_{(20)}$ and $C_{(21)}$ (Sarett, 1949*b*) the Δ_1 value found for our compound should equal the sum of Δ_1 values for each hydroxyl group. Δ_1 at $C_{(3)}$ for a 5:6-unsaturated 3β-hydroxysteroid is −28° (Barton, 1946), Δ_1 at $C_{(20)}$ is −44° for the α-epimer and +111° for the β-epimer (Klyne & Barton, 1949), and Δ_1 at $C_{(21)}$ is 0° (Sarett, 1949*b*). The increment on acetylation calculated for pregn-5-ene-3β:20α:21-triol is −72° (−28 −44 +0) and for the 20β-epimer +83° (−28 +111 +0). The latter value is in agreement with that found for our product which must therefore be pregn-5-ene-3β:20β-21-triol (I).

The preparative oxidations were performed on the compounds II–VII, with a large excess of sodium bismuthate, in 50 % acetic acid. The 20:21-diol (I) was not included in the preparative work since the small quantity of this substance available was reserved for analytical studies. The 17:20-ketol (VII) remained unchanged while the other substances were converted in excellent yields into the products listed in Table 1. The two dihydroxyacetone derivatives IV and V afforded, as was hoped, the corresponding 17-ketosteroids XI and XII. In spite of the large excess of oxidant employed, no effect on any of the nuclear substituents in II–VII was observed.

On the analytical scale, the course and extent of the oxidations were determined by measuring the released formaldehyde and/or 17-ketosteroids. The former was estimated by its colour reaction with chromotropic acid and sulphuric acid (Eegriwe, 1937; cf. MacFadyen, 1945), and the latter were assayed by the Zimmermann reaction (cf. Callow, 1950) with *m*-dinitrobenzene and potassium hydroxide. In order to evaluate the results of the Zimmermann estimations the colorimetric equivalents of the starting materials and of the expected products were determined. The oxidations were performed in the dark with an excess of sodium bismuthate in 5 % acetic acid. Under these conditions good recoveries of formaldehyde and/or 17-ketosteroids from substances I–VI were observed, while the 17:20-ketol (VII) did not yield either product. A comparative series of oxidations with periodate revealed, in agreement with the preparative experiments, the difference in the action of the two oxidants on the dihydroxyacetone derivatives IV and V. For the examples of deoxycorticosterone (DOC, II) and cortisone (IV), the quantities of formaldehyde and/or 17-ketosteroids released on oxidation with bismuthate were directly proportional to the quantity of corticosteroid used. Finally the procedure was applied to a urinary extract: in this case it was found necessary to purify the crude non-volatile oxidation product by Girard separation, prior to the estimation of 17-ketosteroids.

The accuracy and reproducibility of the formaldehyde estimation depends on the rigorous exclusion of daylight during the oxidation with sodium bismuthate: the presence of light evidently catalyses the interaction between bismuthate and aqueous acetic acid to give a product which is a chromogen with the chromotropic-acid reagent, with the consequence of gross and irregular increases in the 'blank' values. The effect of light is greatly weakened if propionic acid is substituted for acetic acid in the oxidation medium. Edwards & Kellie (private communication from Dr A. E. Kellie, Courtauld Institute, Middlesex Hospital, London)

Table 1. *Summary of oxidations with sodium bismuthate*

Nuclear type	Corticosteroid R′			Corticosteroid R″	Product			Product R″
 *A*	CH_2	I	3β:20β:21-Trihydroxypregn-5-ene (20:21-diol)	CH_2OH | $CHOH$ (*a*) | C…H	VIII	17β-Formylandrost-5-en-3β-ol (not isolated)	CHO | C…H
	CH_2	II	21-Hydroxypregn-4-ene-3:20-dione (deoxycorticosterone) (DOC)	CH_2OH | CO (*b*) | C…H	IX	3-Oxoeti-4-enic acid	COOH | C…H
 *B*	CH_2	III	17α:20β:21-Trihydroxypregn-4-en-3-one (triol)	CH_2OH | $CHOH$ (*c*) | C…OH	X	Androst-4-ene-3:17-dione	CO
	CO	IV	17α:21-Dihydroxypregn-4-ene-3:11:20-trione (cortisone)	CH_2OH | CO (*d*) | C…OH	XI	Androst-4-ene-3:11:17-trione (adrenosterone)	CO
	HO—CH	V	11β:17α:21-Trihydroxypregn-4-ene-3:20-dione (Kendall's compound F)	CH_2OH | CO (*d*) | C…OH	XII	11β-Hydroxyandrost-4-ene-3:17-dione	CO
 *C*	CO	VI	3α:17α:20β-Trihydroxy-pregnan-11-one (17:20-diol)	CH_3 | $CHOH$ (*e*) | C…OH	XIII	3α-Hydroxy-5β-androstane-11:17-dione	CO
	CO	VII	3α:17α-Dihydroxypregnane-11:20-dione (17:20-ketol)	CH_3 | CO (*f*) | C…OH		(Unchanged)	

have developed an improved method for the estimation of formaldehydogenic steroids in which propionic acid is advantageously employed.

EXPERIMENTAL

Melting points were determined on a Kofler stage. Microanalyses are by Weiler and Strauss, Oxford. Alumina for chromatography (Hopkin and Williams) was neutralized by treatment with ethyl acetate overnight followed by exhaustive washing with water, and reactivated at 200°.

Of the corticosteroids I–VII (see Table 1), II (as the acetate) and III were kindly provided by British Schering Ltd., V by Merck and Co. Inc. and VII by N. V. Organon. Hydrolysis of commercial cortisone acetate afforded IV, while I and VI were prepared as described below.

Preparations

Pregn-5-ene-3β:20β:21-triol (I). 3β:21-Dihydroxypregn-5-en-20-one 21-acetate (A; R′ = >CH_2, R″ = >CH.CO.CH_2OAc) was supplied by British Schering Ltd.: 60 mg. was refluxed for 1·5 hr. with $LiAlH_4$ (200 mg.) in ether (20 ml.) and the reaction mixture left overnight at room temperature. The excess of reagent was destroyed with ethyl acetate and the mixture worked up in the usual manner. Crystallization of the crude product from acetone yielded the 20:21-diol (I) (25 mg.) as small plates, m.p. 214–224°. Further crystallization raised the m.p. to 221–224°, $[\alpha]_D$ −56° in $CHCl_3$: ethanol (5:1, v/v) (*c*, 0·43). (Found: C, 74·9; H, 10·1. Calc. for $C_{21}H_{34}O_3$: C, 75·4; H, 10·2%.) A sample of m.p. 223–225° showed no depression of m.p. on admixture with Ehrenstein's product (see p. 372) of m.p. 226–228°. The mother liquors of I were evaporated to dryness and the residue (30 mg.) acetylated with pyridine and acetic anhydride on the steam bath (30 min.). The product was chromatographed over 2 g. of Al_2O_3. Elution with CCl_4 yielded first a fraction (10 mg.) which crystallized from methanol as leaflets, m.p. 207–209°: this was not further investigated. Continued elution with the same solvent gave a fraction (19 mg.) which crystallized from methanol as needles, m.p. 182–186°, $[\alpha]_D$ −22° in $CHCl_3$ (*c*, 0·50). (Found: C, 70·8; H, 9·0. Calc. for $C_{27}H_{40}O_6$: C, 70·4; H, 8·8%.) This substance was identical with the product (m.p. 181–185°) of acetylation of the pure 20:21-diol (I). Hydrolysis with 0·5 N-NaOH in aqueous methanol (80%, v/v) afforded pure 20:21-diol (I), m.p. 221–224°.

3α:17α:20β-Trihydroxypregnan-11-one (VI). Hydrogenation of 3α:17α-dihydroxypregnane-11:20-dione (VII) (400 mg.) was effected under the conditions described by Sarett (1949*a*) for the corresponding 3-acetate. The product was acetylated with acetic anhydride in pyridine at room temperature overnight, affording a crude diacetate (480 mg.), m.p. 241–250°. Recrystallization from aqueous acetone gave prisms (321 mg.), m.p. 249–252°: a second crop (86 mg.) had m.p. 235–247°. Hydrolysis of the diacetate (296 mg.) by refluxing with 2 N-KOH in aqueous methanol (90%, v/v) for 1 hr. yielded a product which crystallized from acetone:ether as prisms (161 mg.), m.p. 222–223°. Sarett (1949*a*) found m.p. 220° for VI and m.p. 249–250° for its diacetate.

Preparative oxidations with sodium bismuthate

General procedure. A solution of the steroid (0·14–0·20 m-mole) in 10 ml. of aqueous acetic acid (50%, v/v) is shaken for 30 min. with sodium bismuthate (20 mol. prop.). The reaction mixture is diluted with water (15–20 ml.), treated with sufficient 3 N-KOH to neutralize 75% of the acetic acid, and shaken with benzene (30 ml.). The resulting mixture is filtered with suction: from the filtrate, the benzene layer is separated and the aqueous layer is extracted with portions of benzene (3 × 30 ml.) which have first been used to wash the filter. The combined benzene extracts are washed with water (4 × 20 ml.), each washing being back-extracted with benzene (2 × 5 ml.). The washed extract is evaporated to dryness and the residue purified by a suitable method: examples are given below.

21-Hydroxypregn-4-ene-3:20-dione. The ketol II (57·5 mg., 0·174 m-mole) yielded 55 mg. of a crude crystalline product, m.p. 237–242°. Recrystallization from acetone:light petroleum gave 3-oxoeti-4-enic acid (IX) as dense prisms (43·6 mg.; 79%), m.p. 237–242°. The mother liquor afforded a second crop (6 mg.; 11%) of the same m.p. Sublimation of the product in high vacuum (200°) gave prisms, m.p. 237–241°, $[\alpha]_D$ +161° in $CHCl_3$ (*c*, 1·00). The mixed m.p. with authentic 3-oxoeti-4-enic acid of m.p. 237–244°, $[\alpha]_D$ +159° in $CHCl_3$ (*c*, 0·78), prepared from II by periodate oxidation, showed no depression.

17α:20β:21-Trihydroxypregn-4-en-3-one. The triol III (52·2 mg., 0·15 m-mole) yielded 43 mg. of a crude crystalline product, m.p. 163–171°. Recrystallization from acetone: light petroleum gave androst-4-ene-3:17-dione (X) as prisms (36·7 mg.; 85·5%), m.p. 169–172°. The mixed m.p. with authentic androstenedione of m.p. 170–172·5° showed no depression. A portion sublimed in high vacuum (150°) had m.p. 171–173°, $[\alpha]_D$ +196° in $CHCl_3$ (*c*, 1·61).

17α:21-Dihydroxypregn-4-ene-3:11:20-trione. The dihydroxy acetone IV (56·2 mg., 0·156 m-mole) yielded 47 mg. of a crude crystalline product, m.p. 213–226°. Dissolution in benzene (10 ml.), filtration through Al_2O_3 (1 g.) and elution with 20 ml. more benzene afforded 42·2 mg. (90%) of adrenosterone (XI), m.p. 218–222°. Further elution with benzene (20 ml.) gave 3 mg. (6%) of somewhat less pure material, m.p. 215–225°. Recrystallization from acetone: hexane gave heavy prisms (37·3 mg.), m.p. 224–227°. A sample sublimed in high vacuum (190–200°) had m.p. 223–226°, $[\alpha]_D$ +307° in $CHCl_3$ (*c*, 1·55). (Found: C, 75·7; H, 8·0. Calc. for $C_{19}H_{24}O_3$: C, 76·0; H, 8·0%). The mixed m.p. with an authentic specimen of adrenosterone, m.p. 223–226°, prepared by chromic-acid oxidation of cortisone (Reichstein, 1936), showed no depression.

11β:17α:21-Trihydroxypregn-4-ene-3:20-dione. The dihydroxyacetone V (51·3 mg., 0·142 m-mole) yielded 42 mg. (98%) of a crude crystalline product, m.p. 195–198°. Dissolution in benzene:ether (9:1, v/v; 30 ml.), filtration through Al_2O_3 (1·5 g.) and elution with a further 30 ml. of the same solvent mixture afforded 40·4 mg. (94%) of 11β-hydroxyandrost-4-ene-3:17-dione (XII) as prisms, m.p. 195–197·5°. Recrystallization from ether:light petroleum gave long needles (28 mg.) of the same m.p., $[\alpha]_D$ +219° in $CHCl_3$ (*c*, 1·30). (Found: C, 75·1; H, 8·6. Calc. for $C_{19}H_{28}O_3$: C, 75·5; H, 8·7%.) Reichstein (1937) recorded m.p. 190–192° for this compound.

3α:17α:20β-Trihydroxypregnan-11-one. The 17:20-diol VI (51·3 mg., 0·146 m-mole) yielded an oily product. Dissolution in benzene:ether (9:1, v/v; 10 ml.), filtration through Al_2O_3 (1 g.) and elution with 40 ml. of the same solvent mixture afforded 36 mg. (81%) of 3α-hydroxy-5β-androstane-11:17-dione (XIII), m.p. 185–188°. A sample recrystallized from ether:light petroleum had m.p. 188°: it

readily sublimed in high vacuum (165°) giving crystals of the same m.p.; $[\alpha]_D$ +147°, +148° in ethanol (*c*, 0·59, 0·64). (Found: C, 75·1; H, 9·3. Calc. for $C_{19}H_{28}O_2$: C, 75·0; H, 9·3%). Lieberman, Dobriner, Hill, Fieser & Rhoads (1946) gave m.p. 188–189°, $[\alpha]_D$ +96° in ethanol, for this substance. This value, taken in conjunction with that ($[\alpha]_D$ +145°) given by Lieberman *et al.* (1946) for the corresponding acetate, leads to a change of molecular rotation on acetylation (Δ_1 value) of +210°. The standard Δ_1 value recorded by Barton (1946) for 3α-hydroxy-5β-steroids is +83°: for a number of 11-oxopregnanes studied by Sarett (1949*b*) the mean Δ_1 value was +95°.

Further elution of the Al_2O_3 with 50 ml. benzene:ether (1:1, v/v) afforded an oil (5·6 mg.) which crystallized from ether:light petroleum as heavy prisms (4 mg.) of m.p. 220–223° showing no depression of m.p. on admixture with 3α:17α:20β-trihydroxypregnan-11-one of the same m.p. Thus 8% of pure starting material was recovered.

3α:17α-*Dihydroxypregnane*-11:20-*dione*. The ketol VII (26·5 mg., 0·076 m-mole) yielded a crystalline crude product (26 mg.), m.p. 185–200°. Recrystallization from acetone: light petroleum furnished 19·6 mg. (74%) of unchanged material, m.p. and mixed m.p. 199–203°. The mother liquor afforded a further 2·7 mg. (10%) of less pure ketol VII, m.p. 190–200°.

Use of sodium bismuthate oxidations in analytical work

General procedures

Determination of formaldehyde. Method (*i*) (Conway diffusion procedure). Diffusion is carried out according to Hollander, DiMauro & Pearson (1951). After completed diffusion the contents of the inner chambers of the Conway units are transferred to small test tubes and placed in the oven at 100° for 45 min. The solutions are allowed to cool in the dark and are transferred to rectangular cells (2·5 mm. light path) for measurements at 570 mμ. with the Unicam S.P. 350 spectrophotometer. The instrument is set to zero against a processed blank. The time interval between development and measurement is not critical, provided that the solutions are not exposed to daylight: the extinction values at 570 mμ. remain sensibly constant for at least 1 day (cf. MacFadyen, 1945). The results are evaluated from a standard curve (see Fig. 1). *Method* (*ii*) (direct procedure). A sample of the oxidized solution (0·40 ml.) is mixed directly with 0·80 ml. of the chromotropic acid reagent (cf. Rabinovitch, Decombe & Freedman, 1951). Development of colour and all further operations are performed as in the diffusion process.

Determination of 17-*ketosteroids*. The estimations are performed by the procedure of Zygmuntowicz, Wood, Christo & Talbot (1951), a standard of 20 μg. dehydro*epi*androsterone being included with each series. Measurements are made at 520 and 430 mμ. so that a colour correction (Talbot, Berman & McLachlan, 1942; cf. M.R.C. Committee on Clinical Endocrinology, 1951) can be applied. The results are computed, unless otherwise stated, in terms of the corrected extinction values and are expressed as percentage molar equivalents of dehydro*epi*androsterone.

The colour correction of Talbot *et al.* (1942) was empirically evolved for extracts of acid-hydrolysed urines. Its application to pure steroids has the advantage of reducing the chromogenic contribution of the Δ^4-3-keto grouping in the Zimmermann reaction (see Table 2).

Oxidation with sodium bismuthate. Stock solutions of steroids in absolute ethanol are stored in the dark. Appropriate samples are evaporated in a stream of N_2, and finally *in vacuo*, in 125 × 16 mm. test tubes. Each residue is dissolved in 2·0 ml. of aqueous acetic acid (5%, v/v), 5 mg. of sodium bismuthate (A.R.) is added and the tube shaken for 30 min. with protection from daylight. The mixture is centrifuged and 0·40 ml. samples are taken for the estimation of formaldehyde and/or 17-ketosteroid. The latter determination is performed either (*a*) on the sample used for the estimation of formaldehyde by method (i) by extracting (with $CHCl_3$: methanol, 1:1) the residue remaining in the outer chamber of the Conway unit after completed diffusion, or (*b*) on a separate sample which is evaporated to dryness in a vacuum desiccator over KOH.

Oxidation with periodic acid. Measured quantities of steroids are transferred into glass-stoppered test tubes (approx. capacity 10 ml.) as described above. Oxidations with periodate are performed by the procedure of Hollander *et al.* (1951). From each oxidized solution one sample of 0·40 ml. is taken for the estimation of formaldehyde by the diffusion procedure (method (i)). The remaining volume (1·60 ml.) is extracted with ethylene dichloride (4·0 ml.; purified by shaking with conc. H_2SO_4,, washing with water, aqueous Na_2CO_3 and water, and redistilling) by mechanical stirring for 4 min.: the phases are separated by centrifuging and the aqueous layer is removed with filter paper. The extract is washed successively (by shaking, centrifuging and separating) with 2N-NaOH (1 ml.) and water (0·5 ml.), and is filtered through a small paper. From the filtrate two samples of 1·0 ml. are taken and evaporated to dryness: the residues are assayed for 17-ketosteroids.

Colorimetric equivalents of pure steroids in the Zimmermann reaction

For a single determination, 20–40 μg. of each 17-ketosteroid and 100–200 μg. of each other steroid were taken. The results were calculated from both the corrected and the uncorrected extinction values and are given in Table 2. A few comparative data quoted by other authors are also recorded: strict agreement cannot be expected in view of differences in the colorimetric technique employed.

Influence of light on the reaction between sodium bismuthate and aqueous acetic acid

These experiments were carried out with the collaboration of Miss A. T. Sermin. In preliminary experiments no precautions were taken to exclude daylight during the oxidation of corticosteroids with sodium bismuthate. Under these conditions, the subsequent determinations of formaldehyde were occasionally vitiated by irregular increases in the 'blank' values. The prime cause of the interference was eventually identified as the light-catalysed reaction between aqueous acetic acid and sodium bismuthate. The relevant experimental results are summarized in Table 3.

Bismuthate oxidations of DOC (II) and cortisone (IV) at varying concentrations

Oxidations were carried out by the general procedure with various concentrations (8–64 μg./ml.) of cortisone and DOC. Estimations of formaldehyde and of 17-ketosteroids

Table 2. *Colorimetric equivalents in the Zimmermann reaction*

(Extinctions are expressed as a percentage of that obtained with 1 molecular proportion of dehydro*epi*androsterone (DHA).)

Steroid	Extinctions (% of that for DHA): Measured at 520 and 430 mμ. and corrected	Measured at 520 mμ. only: This paper	Measured at 520 mμ. only: Other data	Ratio $\frac{E_{520}}{E_{430}}$
20:21-Diol (I)	1	4	—	0·8
DOC (II)	10	28	26*	0·8
Triol (III)	13	28	—	0·9
Cortisone (IV)	1	15	25*	0·6
Compound F (V)	3	18	19*	0·7
17:20-Diol (VI)	2	3	—	1·0
17:20-Ketol (VII)	25	27	—	1·6
3-Oxoeti-4-enic acid (IX)	9	22	—	0·8
Androst-4-ene-3:17-dione (X)	118	140	127†	1·5
Adrenosterone (XI)	97	111	—	1·5
11β-Hydroxyandrost-4-ene-3:17-dione (XII)	69	85	—	1·4
3α-Hydroxy-5β-androstane-11:17-dione (XIII)	124	123	123†	2·3

* Private communication from Dr A. E. Kellie. † Wilson (1950).

Table 3. *Blank values in the estimation of formaldehyde*

(Aqueous acetic or propionic acid, 2·0 ml. and $NaBiO_3$, 5 mg. in each experiment; 0·40 ml. samples taken after shaking.)

Solvent	Concn. (%, v/v)	Shaking time (hr.)	Illumination	Extinction at 570 mμ.*
Aqueous acetic acid stored without precautions to exclude light	1·25	1·5	No precautions to exclude light	0·06–0·3
	5	1·5		0·14–0·6
	1·25	1·5	Light excluded	0·035–0·045
	5	1·5		0·09–0·10
	25	1·5		0·22–0·23
Aqueous acetic acid stored in dark	5	0·5	Light excluded	0·045–0·055
	5	0·5	Electric light	0·065–0·070
	5	0·5	Sunlight	1·38–1·42
Aqueous propionic acid stored in dark	5	0·5	Light excluded	0·035–0·045
	5	0·5	Sunlight	0·085–0·095

* Water as blank.

were performed by the two alternative methods, agreement between which is good (Fig. 1). The diffusion procedure (i) is generally to be preferred over the direct method (ii), but the latter is conveniently rapid and appears from these and other experiments to be applicable when pure steroids are being estimated.

Oxidation of cortical steroids and related substances with bismuthate and with periodate

Oxidations of compounds I–VII (50–100 μg./ml.) were performed by the general procedures. The results are summarized in Table 4.

Recovery of formaldehydogenic and 17-ketogenic steroids following addition of triol (III) to a urine extract

These experiments were carried out with the collaboration of Miss A. T. Sermin. A pooled-urine specimen (3·5 l.) was adjusted to pH 1 with HCl and extracted with 1·2 l. $CHCl_3$. The extract was washed successively with 0·1 N-NaOH (2 × 50 ml.) and water (2 × 50 ml.), each washing being back-extracted with $CHCl_3$ (50 ml.). The combined $CHCl_3$ extracts were evaporated *in vacuo* under a stream of N_2 and the residue dissolved in 5% acetic acid (40·0 ml.). Samples of this solution were taken for the following estimations: (1) estimation of formaldehydogenic steroids by method (i) gave $26·2 \times 10^{-8}$ moles/ml.; (2) as in (1) after addition of $6·66 \times 10^{-8}$ moles/ml. of triol III gave $32·5 \times 10^{-8}$ moles/ml., the increase of $6·3 \times 10^{-8}$ moles/ml. represents a recovery of 95% of the triol III; (3) estimation of 17-ketosteroids preceded by separation with Girard T reagent (cf. Pincus & Pearlman, 1941; the procedure was modified by adjusting to pH 7 with phosphate-citrate buffer prior to extraction of the Girard complex) gave $1·75 \times 10^{-8}$ molar equivalents of dehydro*epi*androsterone/ml.; (4) as in (3) but after oxidation with sodium bismuthate by the general procedure gave $4·6 \times 10^{-8}$ molar equivalents of dehydro*epi*androsterone/ml.; (5) as in (4) but after addition of $6·66 \times 10^{-8}$ moles/ml. of triol III gave $10·25 \times 10^{-8}$ molar equivalents of dehydro*epi*androsterone/ml., the increase of $5·65 \times 10^{-8}$ molar equivalents/ml. represents a recovery of 71% of the triol III (see Table 4).

DISCUSSION

The evidence presented here demonstrates the usefulness of sodium bismuthate in the selective oxidative fission of the corticosteroidal side chain for preparative as well as for analytical purposes. The analytical procedure permits the combined estimation of formaldehydogenic and '17-ketogenic' steroids on one sample containing as little as 10 μg. corticosteroid and affords a simple means of differentiating and estimating three groups of corticosteroids: (i) formaldehydogenic, non-ketogenic (types *a* and *b*); (ii) formaldehydogenic and ketogenic (types *c* and *d*); (iii) ketogenic, non-formaldehydogenic (type *e*). A further differentiation between the various side-chain types can be achieved with the aid of the Porter-Silber reaction for dihydroxyacetone derivatives (*d*) (Porter & Silber, 1950), or by employing analytical methods based on the reducing properties of types (*b*) and (*d*) (Talbot, Saltzman, Wixom & Wolfe, 1945; Heard & Sobel, 1946), or by estimating 17-ketosteroids formed from types (*c*) and (*e*) on oxidation with periodate (Talbot & Eitingon, 1944; Fieser *et al.* 1944).

We find our analytical method applicable to urinary extracts: however, the lack of a means of preparing satisfactory extracts has so far severely limited the value of such applications. For the estimation of urinary corticosteroids of the '17-ketogenic' group ((ii) and (iii) above) we prefer a method in which the problems of hydrolysis and extraction are avoided by carrying out oxidation *in situ* with sodium bismuthate (Norymberski, 1952; Norymberski, Stubbs & West, 1953).

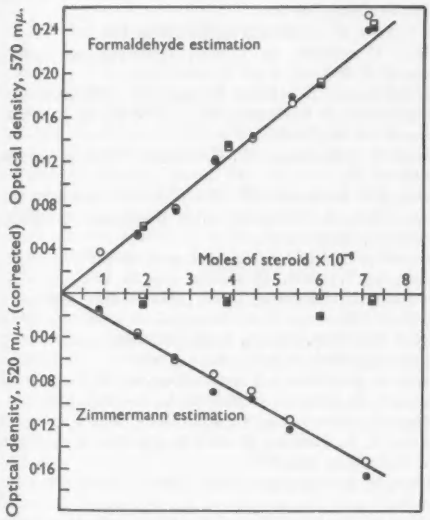


Fig. 1. Bismuthate oxidations of DOC (II) and cortisone (IV).
Upper graph. Formaldehyde estimation method

	(i)	(ii)
DOC	□	■
Cortisone	○	●

Lower graph. Zimmermann estimation method

	(*a*)	(*b*)
DOC	□	■
Cortisone	○	●

SUMMARY

1. Six corticosteroids representing five different side-chain types have been treated with sodium bismuthate in 50% acetic acid. Five compounds were oxidized in excellent yields, whilst a 17-hydroxy-20-ketone remained unchanged.

2. An analytical procedure has been developed based on the estimation of formaldehyde and/or 17-ketosteroids following the oxidation of corticosteroids with sodium bismuthate. The method has

Table 4. *Results of periodate and bismuthate oxidations*

	Recoveries of products in moles %					
	Periodate oxidations			Bismuthate oxidations		
		17-Ketosteroid as DHA			17-Ketosteroid as DHA	
Steroid	Formaldehyde	Found	Expected*	Formaldehyde†	Found‡	Expected*
20:21-Diol (I)	88	11	—	114	7	—
DOC (II)	105	6	0§	93	5	9
Triol (III)	98	115	118	111	116	118
Cortisone (IV)	90	16	0§	98	98	97
Compound F (V)	93	11	0§	91	54	69
17:20-Diol (VI)	12	122	124	8	144	124
17:20-Ketol (VII)	6	34	25	9	30	25

* Based on the colorimetric equivalents (Table 2) of the expected products.
† Determined by method (i).
‡ Determined by method (*b*).
§ Acidic products eliminated during working up.
— = Not measured.

been applied to a urinary extract to which 17α:20β: 21-trihydroxypregn-4-en-3-one (III) was added: reasonable recoveries of formaldehyde and 17-ketosteroid formed from III were obtained.

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Studies on the Proteins of Fish Skeletal Muscle

2. ELECTROPHORETIC ANALYSIS OF LOW IONIC STRENGTH EXTRACTS OF SEVERAL SPECIES OF FISH

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The comparative biochemistry of skeletal-muscle proteins has so far attracted only occasional and incidental attention. From the published work it is possible to make a few comparisons which, however, largely relate either to the fibrillar proteins or particular enzymes of a few types of animal muscle, e.g. rabbit, frog, carp, snail, rat, mouse and insect muscle. In particular, the electrophoretic properties of the fibrillar proteins from different animals are rather similar (Weber & Portzehl, 1952; Hamoir, 1952). Of the proteins which are extractable with dilute salt solutions, only those from frog (Jacob, 1945; Dubuisson & Jacob, 1945), rabbit (Jacob, 1947; Bosch, 1951), carp (Hamoir, 1951) and human (Haan, 1952) muscle have been studied electrophoretically. The derived diagrams show some marked differences between different animals, but the results have been obtained under

such diverse conditions as to make detailed comparisons difficult. In a previous paper (Connell, 1953) suitable conditions for the electrophoretic analysis of fish-muscle protein extracts of low ionic strength have been described, and it was thought worth while to compare extracts from several species of fish under the same conditions.

EXPERIMENTAL

Methods. The conditions of electrophoresis, extraction of the muscle and dialysis have been described previously (Connell, 1953). The extractant was 0·05 *I* buffer (0·0156 M-Na_2HPO_4 + 0·0035 M-KH_2PO_4), pH 7·5 (1 ml./g. mince), and the same buffer was used for equilibrium dialysis and electrophoresis. The recorded pH values are the means of the values of diffusate fluid and protein solution measured at 10°. In the main, the mean pH values varied between 7·5 and 7·6, but in three experiments (68/52, 74/52 and 75/52) they were unaccountably higher than expected.

In some cases (whiting, hake, herring, plaice, lemon sole, turbot and carp) the extracts were quite turbid, and were given a final centrifugation at about 20000 *g* for 15 min before electrophoresis; in others (coalfish, herring, carp) the extract retained a pink colour due to blood or muscle pigments. Analysis of the electrophoretic diagrams by projection, tracing and dividing by perpendicular extrapolation was normally carried out only once.

In some diagrams protein components overlap or completely obscure the δ and ϵ boundaries, rendering accurate evaluation impossible, but an approximate result has been arrived at by the following procedure. It is assumed that the ϵ boundary constitutes 5% of the total area on the descending side, that it remains stationary during the course of electrophoresis, and that it 'spreads' to an extent determined from the average behaviour in experiments without overlapping. All these assumptions are very nearly true as judged from experiments without overlapping. It is then possible to obtain the 'true' areas of the components overlapping the ϵ boundary by subtraction, and thus to evaluate the whole of the descending side. If it is further assumed that the contributions of the components which overlap the stationary boundaries are the same on the ascending as on the descending side, then the area of the δ boundary may be calculated, and consequently the rest of the ascending diagram evaluated. This procedure was adopted with the diagrams of herring, grey skate, thornback skate and cuckoo-ray extracts, and the values for these species in Table 2 must be viewed with this in mind.

Mobilities on the ascending side have been corrected as described previously (Connell, 1953).

Materials. The following twenty species, representing several quite different biological types, have been examined:

Cod (and codling)	*Gadus callarias* L.
Haddock	*G. aeglefinus* L.
Whiting	*G. merlangus* L.
Coalfish	*G. virens* L.
Ling	*Molva molva* L.
Hake	*Merluccius merluccius* L.
Herring	*Clupea harengus* L.
Angler	*Lophius piscatorius* L.
Plaice	*Pleuronectes platessa* L.
Lemon sole	*P. microcephalus* Day
Witch	*P. cynoglossus* Day
Megrim	*Arnoglossus megastoma* Day
Turbot	*Rhombus maximus* Day
Common skate	*Raja batis* L.
Thornback skate	*R. clavata* L.
Cuckoo ray	*R. circularis* Day.
Lesser-spotted dogfish	*Scyliorhinus caniculus* L.
Pike	*Esox lucius* L.
Perch	*Perca fluviatilis* L.
Common carp, mirror carp	*Cyprinus carpio* L.

The names are taken from Jenkins (1936).

The marine species were caught throughout the years 1951–2, a few miles off Aberdeen; the pike and perch were caught in Loch Luichart, Ross and Cromarty, during April 1952; the common and mirror carps were commercial samples of unknown origin. Most of the fish were obtained alive, and in the other cases they were never more than a few hours dead. Unless stated otherwise, the fish were filleted and boned in the commercial fashion and the musculature from both sides used for extraction. In the case of the angler, only the flesh from both sides of the 'tail' was used, whilst only the flesh from the 'wings' of the three skates was used, the tails being omitted. No attempt was made to distinguish the sex or age of the individuals, but none was in any way abnormal in size and appearance.

RESULTS

The results of mobility determinations and of percentage composition are given in Tables 1 and 2, respectively. Occasionally, components failed to resolve in the descending limb and are therefore assessed together, e.g. coalfish, hake, ling, angler. Considerations related to the percentage composition of the diagrams are based on the assumption that the extracts represent essentially similar protein fractions from one species to another. In the case of codling, cod, haddock, dogfish, grey skate, angler, herring, turbot, perch and common carp, more than one individual of each species was examined, and the diagrams from individuals of the same species were found to be substantially the same. In all other cases only one individual was examined. Except with codling and haddock, detailed analysis by division into components was carried out on only one diagram from each species. However, variations between the individuals of one species, as exemplified by the results on codling (Connell, 1953), are small, and it can reasonably be assumed that the variations which occur with other species are also small. The electrophoretic diagrams are depicted in Fig. 1; only the ascending limb is shown. The diagram of both the carp extracts could not be reproduced or accurately traced because of the lack of clarity in the photographic plates, but an approximate tracing of the descending limb of the mirror-carp extract is shown in Fig. 2. Evaluation of the carp diagrams was not justifiable.

Table 1. *Mobilities of fish-muscle components*

(0·05*I* phosphate buffer, 0·5°)

Expt. no.	Species	No. of fish	Mobility (10^5 cm.^{2} v^{-1} sec.$^{-1}$) 1	1*a*	2	3	4	5	6	7	8	9	10
	Codling*	7	10·7±0·45	—	6·7±0·36	5·6±0·37	4·4±0·16	3·6±0·34	2·6±0·41	2·0±0·35	—	—	—
80/52, 80*a*/52	Cod	2†	10·4 (10·3–10·4)	—	6·8 (6·7–6·8)	5·6 (5·5–5·7)	4·5 (4·3–4·6)	3·8 (3·7–3·8)	3·2 (3·0–3·3)	2·2 (2·0–2·3)	—	—	—
		†	10·6 (10·5–10·6)	—	6·9 (6·8–6·9)	5·7 (5·5–5·8)	4·5 (4·4–4·5)	3·4 (3·3–3·5)	2·5 (2·4–2·6)	2·0 (1·9–2·0)	—	—	—
15/52, 16/52, 22/52	Haddock	3	9·2 (8·1–9·8)	—	6·7 (6·1–7·4)	5·2 (4·9–5·6)	4·2 (4·0–4·4)	3·4 (3·3–3·5)	2·6 (2·4–2·8)	1·6 (1·3–1·7)	0·7 (0·6–1·0)	—	—
			8·6 (7·7–9·5)	—	6·2 (5·7–7·0)	5·1 (4·4–5·9)	3·8 (3·5–4·0)	3·2 (2·4–4·3)	2·0 (1·8–2·2)	1·3 (1·1–1·5)	0·7 (0·6–0·9)	—	—
65/52, 65*a*/52	Whiting	2	10·5 (10·3–10·8)	—	6·6 (6·4–6·8)	5·1 (5·1–5·1)	3·7 (3·6–3·8)	3·1 (3·0–3·2)	2·2 (2·0–2·3)	1·4 (1·3–1·5)	—	—	—
			11·1 (11·0–11·2)	—	6·8 (6·7–6·9)	5·4 (5·3–5·4)	3·8 (3·7–3·9)	2·9 (2·8–2·9)	1·8 (1·7–1·8)		—	—	—
73/52	Coalfish	1	10·4	—	6·5	—	4·2	3·6	3·0	2·6	1·5	0·8	—
			10·6	—	6·4	—	4·1	2·7		1·9	0·6		—
66/52	Ling	1	8·4	6·4	5·1	4·3	3·3	3·1	2·3	1·9	—	—	—
			8·8	6·5	5·1	4·1	3·2	2·5	1·6		—	—	—
67/52	Hake	1	10·3	—	6·8	5·4	4·0	3·1	2·5	1·7	—	—	—
			10·3	—	6·4	4·8	3·3	2·3	1·3		—	—	—
64/52, 64*a*/52	Herring	2	10·5 (10·0–11·0)	—	5·5 (5·4–5·6)	4·4 (4·3–4·5)	3·6 (3·6–3·6)	2·5 (2·4–2·5)	1·4 (1·3–1·5)	0·0 (0·0–0·0)	—	—	—
			11·1	—	5·3 (5·2–5·3)	4·1 (4·0–4·2)	3·2 (3·1–3·3)	2·3 (2·2–2·4)	1·5 (1·3–1·6)	0·0 (0·0–0·0)	—	—	—
68/52, 68*a*/52	Angler	2	—	7·5 (7·1–7·9)	5·8 (5·7–5·9)	4·8 (4·7–4·9)	4·0 (3·9–4·1)	3·1 (3·1–3·1)	2·0 (2·0–2·0)	—	—	—	—
			11·0 (10·7–11·3)	—	—	5·1 (5·0–5·2)	3·9 (3·8–3·9)	3·0 (2·9–3·0)	2·0 (1·9–2·1)	—	—	—	—
70/52	Plaice	1	9·3	6·6	5·5	4·3	3·1	2·4	1·6	—	—	—	—
			9·6	6·5	5·3	4·3	3·0	2·3	1·6	—	—	—	—

71/52	Lemon sole	1	9·5	—	5·9	5·3	4·2	3·9	3·2	2·2	1·3	—	—
			10·0	—	5·9	5·1	4·2	3·1		2·3	1·5	—	—
72/52	Witch	1	11·3	—	7·0	6·3	4·8	3·8	3·0	—	—	—	—
			10·9	—	6·8	5·5	4·2	2·9	2·3	—	—	—	—
74/52	Megrim	1	—	—	5·6	5·2	3·7	2·7	2·6	2·1	1·9	—	—
			—	—	5·2	4·4	2·7	2·1	1·7	1·1		—	—
69/52, 69*a*/52	Turbot	2	9·4 (9·1–9·6)	—	6·3 (6·1–6·5)	5·2 (5·0–5·4)	3·7 (3·6–3·8)	2·9 (2·9–2·9)	1·9 (1·8–2·0)		—	—	—
			10·6 (10·0–11·2)	—	6·2 (6·1–6·2)	4·5 (4·3–4·6)	3·4 (3·4–3·4)	2·5 (2·4–2·5)	1·6 (1·5–1·6)		—	—	—
75/52, 75*a*/52	Common skate	2	10·1 (9·7–10·5) 8·9 (8·7–9·2)	6·0 (5·7–6·3)	5·1 (5·0–5·2)	4·5 (4·5–4·5)	3·7 (3·6–3·8)		2·8 (2·6–3·0)	2·3 (2·1–2·5)	0·6 (0·5–0·6)	+0·7‡ (+0·6–+0·7)	+1·7 (+1·6–+1·7)
			9·0 (8·4–9·7)	5·9 (5·7–6·0)	4·7 (4·6–4·8)	2·8 (2·6–3·0)			1·8 (1·6–2·0)		0·6 (0·5–0·6)	+0·7 (+0·6–+0·7)	+1·7 (+1·7–+1·8)
76/52	Thornback skate	1	10·6	6·8	6·2	4·6	4·1	1·9	0·8	+0·4	—	—	—
			—	—	5·3	4·3	3·6	1·6	0·8	+0·4	—	—	—
77/52	Cuckoo ray	1	11·0	6·1	5·6	3·9	3·3	1·9	0·7	0·2	+0·5	—	—
			11·3	6·1	5·3	3·2		1·5	0·5	0·2	+0·5	—	—
61/52, 61*a*/52, 62/52	Dogfish	3	—	6·4 (5·9–7·2)	5·5 (5·3–5·7)	4·3 (4·4–4·6)	3·8 (3·7–3·8)	2·7 (2·7–2·9)	1·5 (1·4–1·7)	1·0 (0·9–1·1)	0·5 (0·4–0·5)	—	—
			9·7	7·7 (7·0–8·1)	5·8 (5·3–6·1)	4·7 (4·4–4·9)	3·4 (3·3–3·5)	2·2 (2·1–2·3)	1·4 (1·3–1·5)	1·0 (0·9–1·1)	0·4 (0·4–0·5)	—	—
39/52	Pike	1	9·6	—	6·8	5·3	4·6	3·4	2·9	1·5	—	—	—
			—	—	6·2	5·3	4·4	2·9	2·2	1·3	—	—	—
40/52, 40*a*/52	Perch	2	11·1 (10·6–11·7)	—	6·6 (6·3–6·8)	5·5 (5·3–5·7)	3·9 (3·8–3·9)	2·6 (2·6±2·6)	1·7 (1·6–1·7)	1·2 (1·1–1·2)	0·6 (0·5–0·6)	—	—
			10·4 (10·4–10·4)	—	6·2 (6·1–6·2)	4·8 (4·7–4·8)	3·4 (3·3–3·4)	1·9 (1·8–1·9)	0·9 (0·7–1·0)			—	—

* Values taken from Connell (1953); arithmetical mean of 'corrected ascending' and descending mobilities ± S.D.
† The first and second lines for each species (except codling) denote the 'corrected ascending' and descending mobilities, respectively. Arithmetical mean; range in brackets.
‡ A plus sign denotes movement towards the cathode.

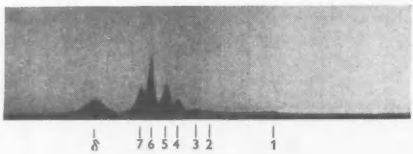
Table 2. *Composition of fish-muscle extracts*

Expt. no.	Species	No. of fish	% total area											
			1	*sp*	1*a*	2	3	4	5	6	7	8	9	10
	Codling*	7	3·5±1·0	5·9±1·2	—	7·6±2·3	6·9±0·8	9·5±1·4	25·4±1·3	30·2±2·9	11·4±1·3	—	—	—
80/52, 80*a*/52	Cod	2† †	1·3 (1·1–1·6)	1·9 (1·6–2·2)	—	3·8 (3·7–3·9)	5·5 (5·3–5·7)	11·0 (11·0–11·1)	21·6 (20·9–22·2)	34·9 (34·7–35·0)	20·0 (19·6–20·4)	—	—	—
			2·9 (2·6–3·3)	3·9 (3·8–3·9)	—	4·3 (3·7–4·8)	6·7 (6·7–6·8)	13·1 (12·9–13·3)	20·8 (20·5–21·0)	32·2 (32·0–32·4)	16·2 (15·1–17·2)	—	—	—
15/52, 16/52, 22/52	Haddock	3	0·5 (0·3–0·7)	1·7	—	3·7 (2·6–4·8)	7·2 (5·1–9·3)	7·9 (7·0–8·7)	33·3 (33·1–33·5)	8·4 (6·7–10·0)	3·9 (3·3–4·4)	34·4 (29·0–39·8)	—	—
			2·7 (2·5–2·8)	2·1	—	5·0 (4·6–5·3)	7·3 (6·1–8·5)	12·6 (10·6–14·6)	28·9 (27·4–30·3)	8·0 (7·8–8·2)	3·3 (2·9–3·6)	31·3 (28·6–34·0)	—	—
65/52, 65*a*/52	Whiting	2	2·1 (2·1–2·2)	6·3 (6·0–6·6)	—	8·1 (7·8–8·3)	7·2 (6·2–8·2)	19·0 (18·8–19·2)	30·8 (30·0–31·5)	16·4 (16·2–16·7)	10·2 (10·0–10·3)	—	—	—
			3·9 (3·7–4·0)	5·7 (4·9–6·4)	—	8·3 (8·3–8·3)	8·9 (8·4–9·4)	20·3 (20·0–20·6)	30·7 (30·5–31·0)	22·3 (20·8–23·7)		—	—	—
73/52	Coalfish	1	1·3	5·1	—	5·8	5·1	3·7	9·0	6·7	44·0	5·3	14·1	—
			1·9	6·0	—	7·0	12·3		19·7		37·6	15·5		—
66/52	Ling	1	3·2	2·3	4·9	6·8	3·2	13·2	33·3	13·8	19·3	—	—	—
			2·8	2·0	3·4	9·4	5·8	15·2	32·7	28·7		—	—	—
67/52	Hake	1	2·4	4·2	—	10·1	14·2	9·8	31·8	6·8	20·7	—	—	—
			3·9	5·9	—	11·3	14·7	16·3	31·3	16·6		—	—	—
64/52, 64*a*/52	Herring	2	0·8 (0·5–1·0)	3·3 (3·1–3·5)	—	8·1 (7·9–8·2)	8·0 (7·9–8·1)	10·7 (10·2–11·1)	26·5 (25·6–27·3)	30·8 (30·4–31·2)	12·0 (11·8–12·3)	—	—	—
			0·7	6·2 (5·7–6·6)	—	8·5 (8·3–8·7)	8·9 (8·8–9·1)	13·0 (12·7–13·2)	24·4 (23·0–25·9)	28·0 (27·8–28·2)	10·7 (6·0–12·3)	—	—	—
68/52, 68*a*/52	Angler	2	—	1·9 (1·5–2·3)	4·9 (4·7–5·0)	7·5 (6·2–8·7)	16·6 (16·5–16·7)	6·7 (6·5–6·8)	37·0 (36·2–37·8)	25·5 (24·0–27·0)	—	—	—	—
			1·5 (0·9–2·0)	20·4 (19·8–21·0)			18·2 (17·9–18·4)	8·7 (8·7–8·7)	31·0 (30·7–31·2)	20·4 (20·3–20·4)	—	—	—	—
70/52	Plaice	1	4·5	3·0	3·6	10·8	5·7	32·8	20·3	19·3	—	—	—	—
			7·2	6·1	6·9	12·2	7·8	32·4	16·6	10·8	—	—	—	—

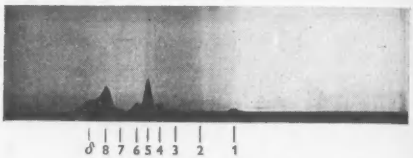
71/52	Lemon sole	1	3·1	3·5	—	6·5	4·6	6·8	21·8	12·7	2·2	38·8	—	—
			4·1	6·4	—	6·0	6·9	14·3	28·0		4·0	30·3	—	—
72/52	Witch	1	4·8	4·8	—	7·9	23·4	8·3	27·4	23·4	—	—	—	—
			2·7	7·3	—	8·9	24·3	10·8	26·7	19·4	—	—	—	—
74/52	Megrim	1	—	1·8	—	5·9	20·3	19·3	4·9	26·5	6·1	16·2	—	—
			—	6·0	—	6·8	16·4	20·2	6·7	23·1	20·8		—	—
69/52, 69*a*/52	Turbot	2	4·9 (4·7–5·1)	2·7 (2·1–3·2)	—	4·6 (4·2–5·0)	20·8 (20·6–21·1)	3·9 (3·3–4·5)	15·5 (14·7–16·3)	45·6 (44·5–46·7)		—	—	—
			5·6 (5·6–5·6)	4·3 (4·0–4·6)	—	8·5 (7·8–9·1)	20·5 (20·0–20·9)	4·5 (4·4–4·5)	17·7 (17·0–18·4)	39·0 (38·4–39·6)		—	—	—
75/52, 75*a*/52	Common skate	2	1·3 (0·7–1·9)	2·9 (2·2–3·7)	4·8 (3·9–5·6)	3·6 (3·2–3·9)	9·9 (9·2–10·6)	3·0 (2·7–3·2)	34·0 (32·4–35·6)		4·9 (4·7–5·1)	8·9 (8·5–9·3)	20·0 (19·0–21·0)	0·8 (0·7–0·9)	6·5 (6·3–6·7)
			4·0 (2·7–5·4)		3·9 (3·6–4·2)	4·5 (4·5–4·5)	8·6 (8·1–9·1)	39·8 (38·6–40·9)			11·9 (11·6–12·2)		20·0 (19·0–21·0)	0·8 (0·7–0·9)	6·5 (6·3–6·7)
76/52	Thornback skate	1	2·0		2·8	3·0	10·8	3·0	16·6	31·5	20·8	9·5	—	—	—
			—		7·3		9·5	2·5	13·8	36·6	20·8	9·5	—	—	—
77/52	Cuckoo ray	1	4·9		6·0	7·9	9·8	21·9	13·3	11·9	24·3			—	—
			4·4		8·5	7·4	6·7	37·0		11·7	11·0	5·5	7·8	—	—
61/52, 61*a*/52, 62/52	Dogfish	3	—		0·8 (0·6–1·1)	1·3 (0·7–2·0)	5·2 (3·5–6·9)	6·4 (5·9–6·6)	21·3 (20·0–23·1)	28·1 (27·9–28·4)	9·1 (8·3–10·1)	2·1 (1·3–3·0)	25·6 (24·2–27·0)		
			0·3?		1·5 (0·9–2·1)	3·9 (3·6–4·2)	6·9 (5·9–7·8)	7·0 (6·9–7·2)	18·7 (18·4–19·2)	24·6 (23·0–26·0)	8·5 (7·0–9·6)	3·6 (3·1–4·0)	25·6 (24·2–27·0)	—	—
39/52	Pike	1	1·7		3·9	—	8·2	4·1	10·3	22·3	43·3	5·5	—	—	—
			—		6·2	—	6·2	3·7	11·2	32·7	35·0	5·0	—	—	—
40/52, 40*a*/52	Perch	2	1·5 (1·3–1·6)		4·6 (3·9–5·2)	—	3·9 (3·6–4·2)	4·2 (3·9–4·4)	23·7 (22·9–24·5)	28·2 (28·0–28·5)	3·3 (2·9–3·6)	12·8 (12·6–13·0)	18·1 (17·7–18·6)	—	—
			1·8 (1·5–2·0)		3·7 (3·5–3·9)	—	5·0 (4·8–5·2)	6·6 (5·8–7·3)	24·9 (23·1–26·8)	31·0 (30·0–31·0)		27·1 (25·6–28·5)		—	—

* Values taken from Connell (1953); arithmetical mean of ascending and descending values ± S.D.

† The first and second lines for each species (except codling) denote ascending and descending values, respectively. Arithmetical means; range in brackets.

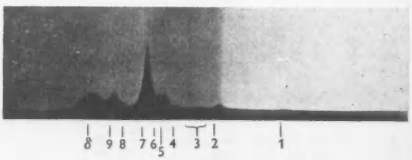


(*a*) Cod, Expt. 80/52.

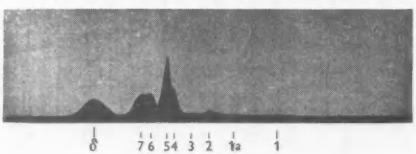


(*b*) Haddock, Expt. 15/52.

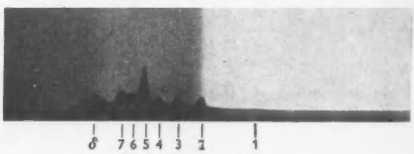
(*c*) Whiting, Expt. 65/52.



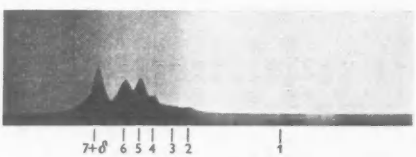
(*d*) Coalfish, Expt. 73/52.



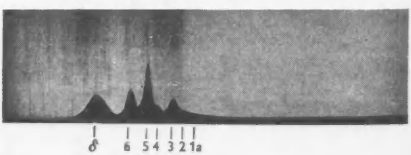
(*e*) Ling, Expt. 66/52.



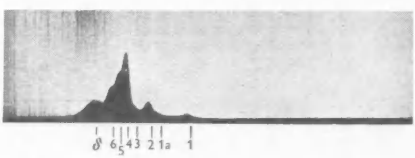
(*f*) Hake, Expt. 67/52.



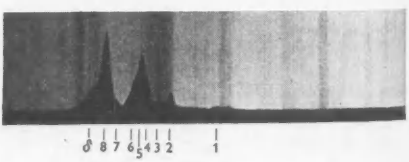
(*g*) Herring, Expt. 64/52.



(*h*) Angler, Expt. 68/52.

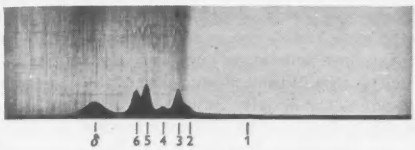


(*i*) Plaice, Expt. 70/52.

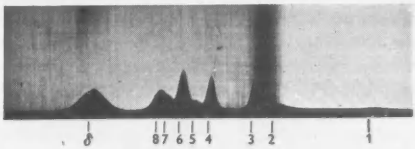


(*j*) Lemon sole, Expt. 71/52.

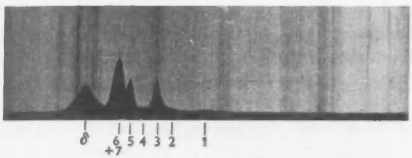
Fig. 1 *a–j*. For legend see p. 385.



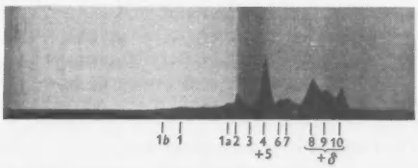
(*k*) Witch, Expt. 72/52.



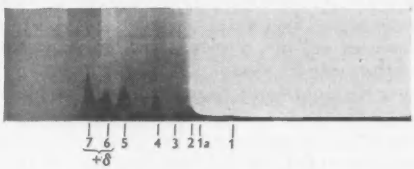
(*l*) Megrim, Expt. 74/52.



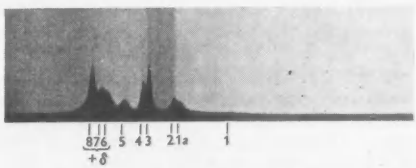
(*m*) Turbot, Expt. 69/52.



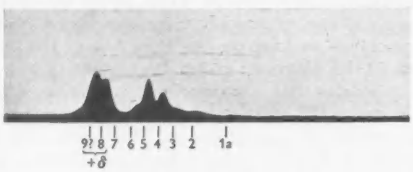
(*n*) Common skate, Expt. 75/52.



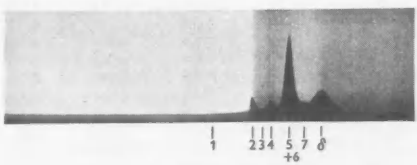
(*o*) Thornback skate, Expt. 76/52.



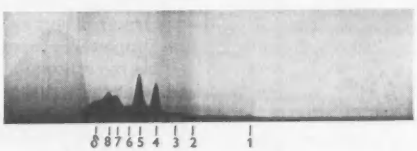
(*p*) Cuckoo ray, Expt. 77/52.



(*q*) Dogfish, Expt. 62/52.



(*r*) Pike, Expt. 39/52.



(*s*) Perch, Expt. 40/52.

Fig. 1 *k–s*.

Fig. 1. Electrophoretic diagrams of fish-skeletal-muscle extracts at 0·05 *I*, pH 7·5. Ascending-limb diagrams only are shown. The direction of migration is from left to right, except in the case of (*n*) and (*r*) where it is from right to left. The diagrams of plaice and pike show the course of electrophoresis at an intermediate stage, because the final photographs were accidentally destroyed. The dark rectangular-shaped patch in the top left-hand corner of some of the diagrams is an aberration in the cell.

The N contents and pH's of the extracts, and the duration and the electrical-field strengths of the experiments are shown in Table 3.

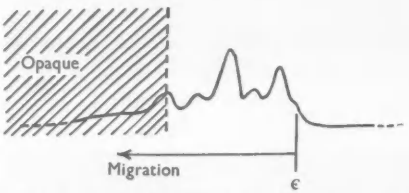


Fig. 2. Approximate tracing of the descending electrophoretic diagram of a muscle extract of mirror carp, 0·05*I*, pH 7·5.

The division of the diagrams into components is somewhat arbitrary and only serves to indicate one possible solution. In some cases it probably over- or under-represents the 'true' picture. This must necessarily lead to errors in both the evaluation of areas and of mobilities, and where considerable overlapping occurs or where components hardly form a 'peak', the values of Table 2 must be taken as denoting only orders of magnitude. The numbering of the components is quite arbitrary and bears no relation to the designations either of Jacob (1947) or of Bosch (1951). As in a previous paper (Connell, 1953), the component with the largest anodic velocity has been designated 1 and the other components numbered consecutively to that with the smallest anodic velocity or largest cathodic velocity. In addition, the number of the component with which the opacity moved has been retained as 2, since it appeared to occupy a special position in all the diagrams studied. This has occasionally resulted in a subdivision of numbering into 1, 1*a* and 1*b* for the faster components.

It is emphasized that components having the same designation in two different species are not necessarily identical, and that Tables 1 and 2 should be read in conjunction with Fig. 1.

Table 3. *N content and* pH*'s of extracts and duration and field strength of the experiments*

Expt. no.	N content (mg./ml.)	pH	Duration (sec.)	Electrical field (v/cm.)
15/52	3·91	7·35	5580	4·95
16/52	3·91	7·35	9360	3·30
22/52	4·28	7·52	4380	4·85
39/52	2·89	7·45	2860	4·85
40/52	2·78	7·48	5520	5·03
40*a*/52	3·00	7·50	5580	5·13
61/52	1·99	7·50	6060	4·88
61*a*/52	2·30	7·53	5580	5·53
62/52	1·99	7·51	5400	5·70
64/52	3·56	7·51	6180	4·99
64*a*/52	3·50	7·53	6000	5·03
65/52	3·55	7·58	4380	6·25
65*a*/52	3·03	7·56	4380	6·25
66/52	3·03	7·61	6280	6·40
67/52	2·74	7·50	4620	6·28
68/52	2·97	7·75	3420	7·04
68*a*/52	3·12	7·69	3720	6·89
69/52	2·85	7·70	3420	6·28
70/52	2·78	7·40	2820	6·66
71/52	4·06	7·45	3900	6·91
72/52	2·03	7·60	3960	6·28
73/52	3·01	7·60	5160	6·28
74/52	4·00	8·02	9240	5·44
75/52	2·69	8·25	4620	6·28
75*a*/52	2·60	7·71	4860	6·28
76/52	2·74	7·58	4440	6·28
77/52	2·69	7·53	3120	7·41
80/52	2·58	7·61	4080	7·41
80*a*/52	2·63	7·62	4200	7·41

DISCUSSION

The most striking fact emerging from this work is that considerable differences in the electrophoretic diagrams of fish-muscle extracts occur from species to species, and that these differences are so characteristic as to 'fingerprint' any one species. These differences are not merely of degree since some quantitatively important components, particularly among the more slowly migrating group, are present in some species but completely absent from others. However, there is an overall similarity in that the components of all the diagrams may be divided into mobility groups, I–III (Jacob, 1947; Connell, 1953), whose quantitative distribution is approximately the same for all diagrams. Thus, group III always consists of one, or possibly more, rapidly migrating components making up less than 4% of the total area of the diagram; group II consists of two or more components quantitatively somewhat larger than group III, and always including component 2 with which the opacity moves. Moreover, 2 always appears in the same relative position. Group I, the slowest group, has the greatest diversity in the number and disposition of components, for example, slow and quantitatively large components (8 and 9) appear in the haddock and coalfish diagrams which are completely absent in cod and whiting diagrams.

This overall similarity has its parallel with the electrophoretic diagrams of different animal plasmas and sera which have been examined by Deutsch & Goodloe (1945) and Moore (1945), respectively. All normal animal plasmas or sera differ in detail from species to species, but are generally similar in showing a large, faster-moving albumin component followed by several smaller, slower globulin components (plus fibrinogen in the case of plasmas) which usually fall into definite mobility groups. This observation has been confirmed with a few electrophoretic analyses of fish-blood sera (Connell, unpublished). Apparently, species differences also occur in the electrophoretic properties of caseins (Dovey & Campbell, 1952).

Whereas with sera the turbidity of the protein mixture migrates with a variable, though limited, number of components (Deutsch & Goodloe, 1945), the largest part of the turbidity of fish-muscle extracts can be associated precisely with one component, which from its position in the diagram appears to be the same in all cases. Generally speaking, the larger the amount of this component, 2, the greater is the opacity of the extract. It has been suggested (Connell, 1953; Jacob, 1947) that this component, or others with similar mobilities, may be a soluble part of the myosin group of proteins, and also that denatured portions of the extract may be associated with it. There is also a very slight increase in turbidity at the level of group III. A curious unexplained feature in the ascending limb is the clearing of the diagram at about the level of the middle components followed by an increase of turbidity towards the δ boundary.

The diagrams of genetically closely related species, e.g. those of the genus *Gadus*, or of *Pleuronectes*, show as much variation amongst themselves as do the diagrams of unrelated species. That it is possible for related species to show special characteristics is demonstrated by the diagram of the skates and perhaps the dogfish, in which, alone among all the species examined, cathodically migrating components are present. Even at pH 8·25 (Expt. no. 75/52), component 10 of the common skate has a considerable cathodic mobility ($+1{\cdot}7 \times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$). This phenomenon may be connected with the uraemic condition of elasmobranch tissues. In addition, the skate diagrams show the greatest electrophoretic complexity yet encountered, the common-skate diagram for example, separating into twelve distinct components.

As far as can be judged, the diagram of common carp is identical with that of the mirror carp (shown in Fig. 2) and both are different from any other species examined. The mirror carp is usually considered to be a variant or a 'sport' of the common carp, so it appears that for the carp such a biological change has no pronounced effect on the muscle proteins examined in this work. On the other hand, Moore (1945) has shown that large electrophoretic differences exist in the serum proteins of two strains of rat, and it is also evident that electrophoretic features of serum proteins may be transmitted from two different species to their hybrid (Brandt, Smith, Andrews & Clegg, 1952).

The diagrams of the freshwater species (pike, perch, carp) do not appear to be markedly different from those of marine species.

There is some evidence to show that age has an effect on the proportions of the electrophoretic components of cod muscle, since the faster components of a cod approximately 90 cm. in length are smaller and the slower components larger, relative to a codling 30 cm. in length. These fish are likely to be about 6 and 2 yr. old, respectively (Graham, 1933). It is not possible to say in this case whether the absolute amounts of any one component are varying because the extractions were not exhaustive. The appearance of the cod diagram is almost identical with that of the previously published codling diagram (Connell, 1953), and the mobility of the components is hardly affected by the difference in age. One observation which may have a bearing on the difference in relative composition is the fact that the amount of myoalbumin (probably represented by group III) in embryonic tissue decreases with development (Crepax, 1952). Differences in age definitely affect the electrophoretic properties and relative proportions of the serum or plasma proteins (Koenig, Perrings & Mundy, 1949; Brandt, Clegg & Andrews, 1951; Beavan, Hoch & Holiday, 1951; Zinsser, 1952).

A number of individual species characteristics may be noted; for example, ling, angler, plaice, the skates and dogfish show a definite component 1*a* which migrates slightly faster than the opacity. Alone amongst the species examined group III of the common skate subdivides into two distinct components 1 and 1*b*. The diagrams of the angler and of the flat fishes, representing rather specialized species, appear to be little different in essence from those of other species. The high proportion of faster components of the witch diagram and their relatively higher mobility, indicates a lower average isoelectric point of the muscle proteins of this species.

The question arises whether any of the components of diagrams of different species are the same. For components which can be easily recognized from diagram to diagram, for example 1, 2 and 3, it seems likely that they are the same in the electrophoretic sense that the serum albumins of different species are the same. The serum albumins invariably occupy the same position in the electrophoretic diagram, although there are marked differences in the mobilities from species to species, even under identical conditions. This is true also of components 1, 2, 3 and possibly 4 of the muscle diagrams. In the more complex part of the diagrams, group I, it is impossible to make conclusions about identity. The most that can be said about group I is that if any of the components in it are identical then their amounts must vary enormously among the species, and that components present in one species are completely absent in others.

The extracts which have been studied in this work contain the largest proportion of the known enzymes of muscle, so that species differences in the derived electrophoretic diagrams may indicate special physiological differences between fish, for example, the fact that arginase is present in elasmobranch but normally not in teleost muscle.

SUMMARY

1. Muscle extracts of twenty different species of fish have been examined electrophoretically at 0·05*I*, pH 7·5.

2. Each species has a characteristic electrophoretic diagram, and considerable differences in mobility and composition exist between species.

3. The diagrams may, however, be still divided into definite mobility groups of approximately consistent quantitative size.

4. The diagrams of related species appear to be as dissimilar as those of unrelated species.

5. The effect of age and of species variants on the diagrams has been examined.

6. Elasmobranch muscle appears to contain proteins with unusually high isoelectric points.

7. The question of the identity of components of different diagrams has been discussed. Whatever the answer, it is certain that components which are present in some species are completely absent in others.

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Comparative Observations on the Chemical Properties and Biological Activities of L-Ascorbic and D-*iso*Ascorbic Acids

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In a previous publication the course of oxidation of L-ascorbic acid under various conditions and in the presence of different accompanying substances was described (Leibowitz & Guggenheim, 1938*a*). It was shown that the stability of the vitamin in solution depends specifically on the nature of the accompanying substances and on the pH. It has also been shown that L-ascorbic acid exerts a detoxifying action on potassium cyanide, phenol and tetanus toxin (Leibowitz & Guggenheim, 1938*b*; Kligler, Guggenheim & Warburg, 1938). Some evidence, derived from titrimetric and cryoscopic data, indicated that the detoxifying effect results from the reversible formation of a chemical compound between the vitamin and potassium cyanide or phenol. Other authors have reported that ascorbic acid diminishes the toxicity of various other substances such as arsphenamines, lead, sulphonamides, benzene, trinitrotoluene, and anaesthetics (Bicknell & Prescott, 1948). It has been tentatively suggested that the antiscorbutic function of L-ascorbic acid may be at least partially due to its ability to detoxify naturally produced toxic substances (Leibowitz & Guggenheim, 1938*b*).

Numerous clinical and experimental observations have indicated that ascorbic acid plays a role in certain forms of anaemia (Vilter, 1947; Dyke, Della Vida & Delikat, 1942; May, Nelson, Lowe & Salmon, 1950). Studies by Nichol & Welch (1950) and Welch, Nichol, Anker & Boehne (1951) have

shown that ascorbic acid promotes the formation of citrovorum factor from folic acid.

A series of chemical analogues of L-ascorbic acid has been prepared synthetically (Smith, 1946). One of these compounds, *iso*ascorbic acid (D-araboascorbic acid) shows great chemical similarity to vitamin C. This compound, however, has only one-twentieth of the antiscorbutic activity of L-ascorbic acid (Dalmer & Moll, 1933; Demole, 1934). These facts afforded the opportunity of testing our hypothesis concerning the relationship between the antiscorbutic activity of vitamin C and the above-mentioned properties. The two compounds were compared as to: (1) their ability to form complexes with potassium cyanide or phenol; (2) their detoxifying effect on potassium cyanide, phenol and tetanus toxin; and (3) their ability to increase urinary excretion of citrovorum factor after administration of folic acid.

MATERIALS AND METHODS

The ability of ascorbic acid and of *iso*ascorbic acid to detoxify potassium cyanide was tested with *Escherichia coli*. The bacteria were grown in nutrient broth, and the amount of growth obtained after 24 hr. was measured turbidimetrically in a Klett-Summerson photoelectric colorimeter. Tetanus toxin was prepared in the following way: *Clostridium tetani* was grown in Tarozzi broth at 37°. After 10 days the broth was filtered and centrifuged, and the toxin-containing supernatant was tested on young mice weighing 15–25 g. Appropriately diluted broth was injected intramuscularly allowing 0·1 ml./10 g. of body weight. The mice were observed for 6 days and the mortality from tetanus noted.

The conversion of folic acid into citrovorum factor was studied with six adult rats weighing 200–300 g. They received a basal diet consisting of corn starch 73, casein 18, olive oil 5, and salt mixture 4%, which was supplemented with 0·3 mg. riboflavin, 0·2 mg. thiamine, 0·1 mg. pyridoxine, 1·6 mg. calcium pantothenate, and 100 mg. choline chloride per 100 g. ration. Each rat received 100 i.u. vitamin A and 4 i.u. vitamin D twice weekly. Citrovorum factor was determined with *Leuconostoc citrovorum* (ATCC 8081) according to Sauberlich & Baumann (1948). After incubation for 3 days at 37° the acid produced was titrated with 0·02 N-NaOH. Synthetic citrovorum factor (leucovorin)* in the form of the hydrated barium salt was used as standard.

RESULTS

Polarimetric studies

Experiments on the change in the rotation values of ascorbic acid and *iso*ascorbic acid induced by potassium cyanide will be described elsewhere. These experiments require special treatment because of the complications arising from the powerful effect of pH on the optical activities of both acids. Data typical of many experiments with phenol are presented in Table 1. Small but significant changes are induced in the optical rotations of both ascorbic acid and *iso*ascorbic acid, the dextrorotation of the former being slightly decreased and the laevorotation of the latter slightly increased. The order of magnitude of the effects is almost the same in both cases. The effects are immediate but are somewhat intensified by incubation at 30°.

Table 1. *Effect of phenol on the optical rotation of ascorbic and* iso*ascorbic acids in aqueous solutions*

[Ascorbic acid] (M)	[*iso*Ascorbic acid] (M)	[Phenol] (M)	Incubation at 30° (hr.)	α_D (1 dm.) (°)
0·1	—	—	0	+0·39
0·1	—	—	6	+0·38
0·1	—	0·1	0	+0·36
0·1	—	0·1	6	+0·36
0·1	—	0·1	24	+0·34
0·4	—	—	0	+1·79
0·4	—	—	6	+1·77
0·4	—	—	24	+1·78
0·4	—	0·1	0	+1·74
0·4	—	0·4	0	+1·70
0·4	—	0·4	6	+1·71
0·4	—	0·4	24	+1·70
—	0·1	—	0	−0·28
—	0·1	—	6	−0·26
—	0·1	0·1	0	−0·30
—	0·1	0·1	6	−0·30
—	0·1	0·1	24	−0·31
—	0·4	—	0	−1·09
—	0·4	0·4	0	−1·15
—	0·4	0·4	6	−1·18
—	0·4	0·4	24	−1·17

Cryoscopic studies

The freezing-point depressions resulting from the mixing of equal amounts of equimolecular aqueous solutions of ascorbic acid or *iso*ascorbic acid with potassium cyanide or phenol were measured. The freezing-point depressions of the mixtures were measured immediately after mixing. The half-values of the sums of the depressions of the components were taken as 'calculated' values (Table 2).

The experimental values of the freezing-point depressions of mixtures of each of the acids with potassium cyanide or phenol were appreciably less than those calculated from separate depressions of the components, indicating a decrease in molecular concentration, due to partial combination of the acids with the reagents to form condensation or addition products. The effect was more pronounced with potassium cyanide than with phenol. No further change in the depression values was induced by incubation of the mixtures at room temperature or at 30° for 24 hr.

* Leucovorin was supplied by the Lederle Laboratories Division, American Cyanamid Company, by the courtesy of Dr T. H. Jukes.

Table 2. *Effect of potassium cyanide and phenol on cryoscopic determinations in solutions of ascorbic and* iso*ascorbic acids*

[Ascorbic acid] (M)	[*iso*Ascorbic acid] (M)	[KCN] (M)	[Phenol] (M)	Δ_{Found} (°)	$\Delta_{Calculated}$ (°)
0·1	—	—	—	0·203	—
—	0·1	—	—	0·210	—
—	—	0·1	—	0·354	—
0·05	—	0·05	—	0·257	0·278
—	0·05	0·05	—	0·262	0·282
0·2	—	—	—	0·402	—
—	0·2	—	—	0·412	—
—	—	—	0·2	0·389	—
0·1	—	—	0·1	0·385	0·396
—	0·1	—	0·1	0·393	0·401

Table 3. *Effect of ascorbic and* iso*ascorbic acids on the potassium cyanide inhibition of* Esch. coli

(The figures indicate galvanometer readings obtained after 24 hr. incubation of bacteria with solutions of KCN, ascorbic acid and *iso*ascorbic acid. Readings adjusted to 200 for culture without substances added. Average of three sets of experiments.)

			Substances		
[KCN] (M)	[Ascorbic acid] (M)	[*iso*Ascorbic acid] (M)	Freshly dissolved	Dissolved and mixed together 24 hr. before the onset of incubation	Dissolved and kept separately for 24 hr. before the onset of incubation
—	—	—	200	200	200
—	0·01	—	258	254	255
—	—	0·01	243	212	222
0·02	—	—	0	25	25
0·01	—	—	54	81	81
0·005	—	—	71	102	102
0·02	0·01	—	0	141	54
0·01	0·01	—	51	176	118
0·005	0·01	—	77	192	113
0·02	—	0·01	10	91	23
0·01	—	0·01	33	139	103
0·005	—	0·01	93	155	125

Detoxication of potassium cyanide

Three experiments were performed. In the first experiment neutralized solutions of potassium cyanide, ascorbic acid and *iso*ascorbic acid were added to samples of nutrient broth in amounts to give the final concentration indicated in Table 3. Sufficient water was added to bring the volume to 5 ml.; then the test tubes were inoculated with *Esch. coli*. In the second experiment similar mixtures of potassium cyanide, ascorbic acid and *iso*ascorbic acid solutions were incubated at room temperature for 24 hr. before being added to the nutrient broth. In the third experiment which served as control for the second, the solutions of potassium cyanide, ascorbic acid and *iso*ascorbic acid were kept separately at room temperature for 24 hr. and separately added to the nutrient broth immediately before inoculation. The amount of growth obtained after 24 hr. incubation is shown in Table 3.

It may be observed that neither ascorbic acid nor *iso*ascorbic acid was able to affect the potassium cyanide inhibition of growth of *Esch. coli* when added separately immediately prior to incubation. If, however, the ascorbic acid or *iso*ascorbic acid were pre-incubated with potassium cyanide for 24 hr., the mixtures were far less toxic for the bacteria than the same concentrations of potassium cyanide alone. These findings confirm previous results (Leibowitz & Guggenheim, 1938*b*) obtained with ascorbic acid. It is noteworthy that no marked difference was noted between the detoxifying effect of ascorbic acid and *iso*ascorbic acid.

Detoxication of phenol

Solutions of phenol (50 mg./ml.) were incubated with equimolar quantities of ascorbic acid or *iso*-ascorbic acid at 40° for 4 hr. The toxicity of these solutions was tested on young rats weighing 50–100 g.; the dose injected subcutaneously contained 50 mg. phenol per 100 g. of body weight. Three groups, each comprising twenty-two animals were used: the first group received phenol-ascorbic acid, the second phenol-*iso*ascorbic acid, the third phenol alone. In the first group six rats died, in the

second twelve, whereas in the third group all twenty-two animals succumbed. The difference in mortality between the first or the second group and the third group proved to be statistically significant, whereas the difference between the first and the second group was insignificant. Ascorbic acid and *iso*ascorbic acid are, therefore, equally active in detoxifying phenol.

Detoxication of tetanus toxin

Equal volumes of the toxic broth and of solutions of ascorbic acid or *iso*ascorbic acid (1 mg./ml.) were incubated at 37° for 24 hr. In the first experiment the broth was diluted 1:100 and injected into ten mice. All these mice died during the next 6 days. Control groups given the toxin-ascorbic acid or the toxin-*iso*ascorbic acid mixture suffered no loss during the same time. In the second experiment two groups of thirty mice were injected with a solution diluted 1:8 of the toxic broth which had been incubated with ascorbic acid or *iso*ascorbic acid. The mortality during the following 6 days amounted to twenty and twenty-one mice, respectively. Thus no difference was noted in the ability of ascorbic acid and *iso*ascorbic acid to detoxify tetanus toxin.

Conversion of folic acid to citrovorum factor

Rats were maintained for 6 days on the normal diet. During the following 6 days the rats received 1 mg. folic acid daily intraperitoneally. During the third and fourth 6-day periods the rats received daily injections of folic acid and 17·6 mg. of ascorbic acid or *iso*ascorbic acid. Urine was collected and analysed for citrovorum factor during the last 3 days of each period. Table 4 shows the average daily excretion of citrovorum factor during these four periods.

Table 4. *Urinary excretion of citrovorum factor by rats*

(Average of six rats; means and standard errors.)

Substances injected	Citrovorum factor in urine (μg./day/rat)
None	2·9 ± 0·32
Folic acid	13·5 ± 5·38
Folic acid + ascorbic acid	142·0 ± 31·25
Folic acid + *iso*ascorbic acid	153·0 ± 14·58

It follows from Table 4 that both ascorbic acid and *iso*ascorbic acid increase urinary excretion of citrovorum factor. *iso*Ascorbic acid was found to be as active in this respect as ascorbic acid.

DISCUSSION

The similarity which exists between the chemical properties of ascorbic acid and *iso*ascorbic acid extends to their biological activities as well. The two compounds have almost the same ability to combine with potassium cyanide or phenol as measured polarimetrically or cryoscopically. Moreover, both ascorbic acid and *iso*ascorbic acid prevent the toxic effects of potassium cyanide, phenol and tetanus toxin on *Esch. coli*, rats, and mice, respectively. Ascorbic acid and *iso*ascorbic acid also show practically the same effect on the conversion of folic acid into citrovorum factor in rats. Identical actions of ascorbic acid and its analogues on other biological systems have already been described. L- and D-Ascorbic acids are equally effective in increasing the height of contraction of the isolated frog heart (Krayer, Linstead & Todd, 1943) and in reducing the toxicity of neoarsphenamine (McChesney, Barlow & Klinck, 1944). Ascorbic acid and *iso*ascorbic acid exert the same effect in promoting the oxidation of *p*-hydroxyphenylpyruvic acid (Knox & LeMay-Knox, 1951) and of tyrosine by liver homogenates (Sealock, Goodland, Summerwell & Brierly, 1952). *iso*Ascorbic acid can replace ascorbic acid as growth factor for *Trichomonas* (Cailleau, 1939) but not for trypanosomes (Lwoff, 1951).

The role played by ascorbic acid in the metabolic activation of folic acid has been studied by Welch and co-workers (Nichol & Welch, 1950; Welch *et al.* 1951). They found that the addition of either ascorbic acid or its analogue glucoascorbic acid to liver slices *in vitro* markedly increases the conversion of folic acid to citrovorum factor, and that both substances increase the excretion of citrovorum factor when administered together with folic acid. On the other hand, such potent reducing agents as cysteine and glutathione had little or no influence.

Our experiments show that *iso*ascorbic acid shares with ascorbic acid the ability to detoxify potassium cyanide and phenol and to stimulate the conversion of folic acid to citrovorum factor in the rats. Since the antiscorbutic activity of *iso*ascorbic acid is negligible, it seems impossible to assume that the antiscorbutic activity of ascorbic acid is due to the above-mentioned chemical and biological properties.

SUMMARY

1. Ascorbic and *iso*ascorbic acids (D-araboascorbic acid) show similar abilities to combine with potassium cyanide and phenol in aqueous solutions.

2. Ascorbic and *iso*ascorbic acids are equally effective in detoxifying potassium cyanide, phenol and tetanus toxin, and in the conversion of folic acid into citrovorum factor.

3. The antiscorbutic activity of ascorbic acid cannot be explained on the basis of the detoxifying activity of this substance.

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The Effect of L-Ascorbic Acid on the *in vitro* Activity of Polyphenoloxidase from Potato

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In recent years many studies of the action of polyphenoloxidase on various substrates, *in vitro*, have been carried out in the presence of L-ascorbic acid (Asimov & Dawson, 1950; Miller, Mallette, Roth & Dawson, 1944; Robinson & Nelson, 1944; Sreerangachar, 1943). In these studies the L-ascorbic acid has been assigned the role of a reducing agent for any *o*-quinones produced during the oxidation of the substrates. It has been generally assumed (Asimov & Dawson, 1950; Miller & Dawson, 1941; Kendal, 1949; Sreerangachar, 1943) that the ascorbic acid has no other effect on the system, although many observers have reported that the rate of oxidation was sometimes significantly different in the presence of ascorbic acid than in its absence (Kendal, 1949; Roberts & Wood, 1950; Krueger, 1950).

Krueger (1950) made a study of the effect of ascorbic acid on the rate of oxidation of tyrosine by mushroom polyphenoloxidase and concluded that initially the ascorbic acid had a 'pro-oxidant' effect on the system since the initial oxygen uptake was substantially increased and the induction period was decreased (cf. Kendal, 1949), but later in the reaction it had an antioxidant effect since the rate of production of L-β-3:4-dihydroxyphenylalanine (dopa) was decreased below that for the ascorbic-acid-free system.

The investigations of Kertesz (1951, 1952) have shown that the observed oxidation rates of polyphenoloxidase systems in the presence of ascorbic acid are markedly dependent on the concentration of cupric ion, and since Krueger apparently did not use copper-free water for his experiments, it seemed worth while to re-investigate this system.

Our experiments, using a colorimetric procedure to determine enzyme activity, show that ascorbic acid has an inhibitory action on potato polyphenoloxidase which is not elicited in the presence of ionic copper. However, when the enzyme was treated with ascorbic acid in the absence of oxygen and the ascorbic acid subsequently removed by dialysis, it was irreversibly changed so that reactivation with cupric salts was not possible. Oxidation products of ascorbic acid, including dehydroascorbic acid, were found to have little effect on the enzyme.

It is suggested that the ascorbic acid affects specific structures of the enzyme (cf. Kertesz, 1951, 1952) in which copper is involved.

EXPERIMENTAL

Materials

Reagents. All salts used were of A.R. quality and distilled water freed from copper (lowered from 95 μg./l. to undetectable amounts) by passage through a mixed-bed ion-exchange resin column (Amberlite MB 1) was used throughout.

Polyphenoloxidase. Potato peelings (50 g.) were macerated in a Waring blendor with ice-cold water (50 ml.), the mixture centrifuged, the supernatant liquid treated with one-third of its volume of acetone (at $-20°$) and the precipitate discarded. The enzyme was precipitated by the addition of more acetone (final concentration 50%, v/v), taken up in 10 ml. of ice-cold water, centrifuged, and the supernatant stored at 0°. It was diluted before use.

Ascorbic acid solution. This was freshly prepared for each experiment.

Dehydroascorbic acid. This was prepared according to Mapson (1946), and its concentration determined by the microbiological method of Mapson & Ingram (1950).

Measurement of enzyme activity

Colorimetric method. The enzyme solution, ascorbic acid or other constituents and 0·1 M-phosphate-citrate buffer (McIlvaine) (determined pH, 5·98) to give a volume of

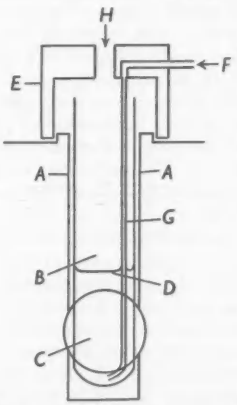

Fig. 1. Diagram of apparatus. *A*, spectrometer case; *B*, 0·5 in. test tube; *C*, light source and photocell perpendicular to plane; *D*, liquid level; *E*, air-lead cap; *F*, air inlet; *G*, hypodermic tubing; *H*, hole for addition of substrate, etc.

3·5 ml. were placed in a 0·5 in. diameter tube of a Unicam D.G. spectrophotometer, and the air-lead cap (Fig. 1) placed in position. Air was bubbled through the mixture at a constant rate (700 ml./hr.) and the galvanometer adjusted to read 100% transmission at 480 mμ. ($E = \log I_0/I = 0$). Catechol (0·5 ml.; 4 mg./ml.) was quickly introduced from a hypodermic syringe (final volume, 4·0 ml.) and readings taken every 15 or 30 sec. All results for each series of experiments were obtained in the shortest possible time since no temperature control was possible. Experimental results were reproducible with a maximum error of $\pm 2\%$.

Although the passage of air bubbles caused slight oscillation of the galvanometer, the effect was constant and readings could be taken with ease. Interruption of the air flow was not nearly so satisfactory because of galvanometer drift. The rate of air flow specified was found to be the most satisfactory, lower rates being unsteady while much higher rates caused excessive foaming, although this could be avoided by using methylheptanol. To ensure uniformity of air flow, stainless steel hypodermic tubing (int. diam. 0·3 mm.) was used as a capillary lead throughout these experiments, and had no apparent effect on the system.

Sreerangachar's (1943) *method.* Enzyme, ascorbic acid and buffer solution were equilibrated in tubes (200 × 25 mm.) under nitrogen for 10 min. at $35° \pm 0·1°$, catechol introduced (total vol. 10 ml.) and air drawn through at 8 l./hr. (Moores, Greninger & Rusoff, 1951). After 5 min. an aliquot of the solution was added to excess 1% oxalic acid and the ascorbic acid determined colorimetrically (Ponting & Joslyn, 1948).

RESULTS

Investigation of the colorimetric method for the measurement of enzyme activity

The shape of the curve. Using the colorimetric method, Ponting & Joslyn (1948) and Warner (1951) stated that the plot of extinction against

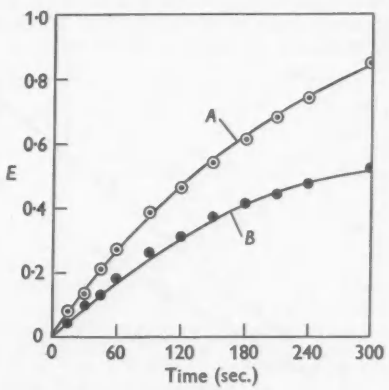

Fig. 2. The increase of extinction with time in the catechol-polyphenoloxidase system, showing the distinct curvature of the plot. Vol. of enzyme used: *A*, 1·0 ml.; *B*, 0·5 ml. Catechol (2 mg.) and total vol. (4·0 ml.) in each case.

time in a catechol-polyphenoloxidase system gave a straight line, although the latter author found that this relationship held only for the first 2·5 min. period. In our hands these results held true when using low enzyme concentrations giving extinction differences of 0·01–0·05/min. (cf. Ponting & Joslyn, 1948). With the concentrations we have used for our work, however, the plot usually had a distinct curvature, as can be seen from Fig. 2, in which two different enzyme concentrations are used. This

corresponds to the findings of Wallerstein, Alba & Hale (1947), who used macerated potato tissue and catechol.

It was shown by Miller *et al.* (1944), using the chronometric technique, in which the time taken for small amounts of ascorbic acid added to the enzyme-substrate system to be oxidized is measured, that during the first 2 or 3 min. of oxidation of catechol, catalysed by polyphenoloxidase, *o*-benzoquinone is produced according to the equation

$$Q = at/(b+t), \qquad (1)$$

where Q is the concentration of quinone, t is the time in sec., and a and b are constants determined by the extent of enzyme inactivation during the initial stage of the reaction. The values of a and b which determine the curvature of the reaction course may be evaluated from the slope and the intercept of the linear curve obtained from the reciprocal equation

$$\frac{1}{Q} = \frac{b}{a}\left(\frac{1}{t}\right) + \frac{1}{a}, \qquad (2)$$

and since the initial rate of reaction $dQ/dt(t \doteqdot 0)$ is given by

$$dQ/dt = a/b, \qquad (3)$$
$$(t \doteqdot 0)$$

it is thus equal to the reciprocal of the slope of the linear curves obtained from equation 2. Miller *et al.* (1944) evaluated the quinone concentration to the

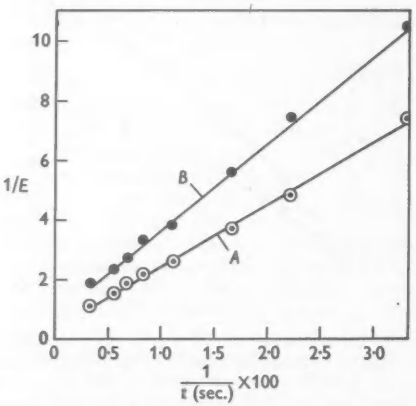

Fig. 3. The results of Fig. 2 shown as a reciprocal plot.

ascorbic acid oxidized by the system. A consideration of the proposed mechanism of the oxidation of catechol by polyphenoloxidase (Nelson & Dawson, 1944) suggests that, since the transformation of the initial oxidation product is independent of enzyme action and virtually instantaneous, the quinone produced can be related directly to absorbance. Consequently, a plot of $1/E$ (E = extinction) against $1/t$ should produce a straight line. This is confirmed by Fig. 3 where the results obtained from Fig. 2 have been plotted in this way. Hence the initial rates of reaction can readily be determined.

Miller *et al.* (1944) showed that the initial rate of reaction could also be deduced from the equation

$$\frac{a}{b} = \left(\frac{1}{t_1} - \frac{1}{t_2}\right) \Big/ \left(\frac{1}{Q_1} - \frac{1}{Q_2}\right). \qquad (4)$$

A comparison of the rates derived from the slopes of the reciprocal curves (Fig. 3), calculated from equation 4 ($Q = E$) and estimated from the original curves (Fig. 2) is shown in Table 1.

Table 1. *Initial rate of oxidation of catechol by potato polyphenoloxidase*

(In each experiment 2 mg. of catechol were used, in total vol. 4 ml. Initial rate given as increase in E/min.)

Enzyme solution (ml.)	Initial rate		
	From Fig. 2	From Fig. 3	From equation 4
0·5	0·18	0·187	0·190
1·0	0·27	0·275	0·284

Since, however, the initial rates obtained from the plots of absorbance against time give satisfactory agreement with those derived from both the reciprocal plots and from equation 4 they have been used throughout and are designated R (graph).

In using the extinction as a measure of *o*-benzoquinone produced we have assumed that the same coloured condensation product is produced regardless of conditions. Warner (1951) has pointed out that in his system the colour produced changed progressively from red-brown to bright yellow with increasing substrate concentration. He was working with relatively crude enzyme preparations, and indicated that the colour was probably due in part to secondary reactions between the oxidation products of catechol and other compounds such as amino acids present in his system (cf. Jackson & Kendal, 1949; Trautner & Roberts, 1950). We have found that crude enzyme preparations from different sources give easily observed differences in colour with catechol (unpublished results). However, with the enzyme used here the absorption maximum of the colour product was always at 480 mμ., and it may be concluded that our assumption was justified.

The effect of the rate of air flow. The effect of the rate of air flow through the system is shown in Fig. 4. At the two lower rates of air flow (440 and 700 ml./hr.; curve *A*) the result was found to be virtually the same (cf. Asimov & Dawson, 1950). At the highest rate of flow (Fig. 4, curve *B*) the initial rate of enzyme activity is slightly higher, but as can be seen the rate of increase of absorbance falls off more rapidly than at the lower rates of air flow. This may

be due to surface denaturation of the enzyme by the air stream (Tenenbaum, 1940), a possibility which is rendered more likely by the observed diminution of the activity of a polyphenoloxidase solution through which air has been passing (1500 ml./hr.) for 2 hr. (Fig. 4, curve *C*). The initial rate of reaction determined without air flow is virtually the same (Fig. 4, curve *D*), but the rate falls off considerably after 1·5–2 min., and an examination of the reaction tube after 10 min. showed that there was a dark-brown top layer. Careful mixing of the two layers gave no

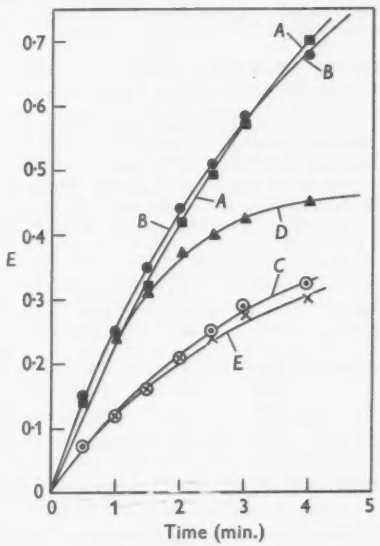

Fig. 4. Effect of rate of air flow on the measurement of enzyme activity. *A*, air passed at either 440 or 700 ml./hr.; *R* (graph) = 0·32 *E*/min.; *B*, air passed at 1440 ml./hr.; *R* (graph) = 0·36 *E*/min.; *C*, air passed at 700 ml./hr. using enzyme solution which previously had air at 1500 ml./hr. passing through for 2 hr.; *R* (graph) = 0·125 *E*/min. *D*, no aeration, but enzyme and substrate solutions completely mixed initially; *R* (graph) = 0·32 *E*/min. *E*, no aeration, but enzyme and substrate solutions incompletely mixed initially; *R* (graph) = 0·12 *E*/min. In each case 0·5 ml. enzyme and 2 mg. catechol in a total volume of 4·0 ml. were used.

significant increase in absorbance even after some minutes, indicating that the oxygen tension of the solution was too low for enzyme action to take place (cf. oxidation of ascorbic acid in boiling water, Mapson, 1941). This was confirmed by subsequent passage of air through the solution when the total absorbance was raised to almost the same level as curve *A*. Curve *E* (Fig. 4) shows the result of incomplete mixing. In this case, the lower half of the reaction tube was virtually colourless.

From these experiments it can be seen that although there is no need to aerate the solution in order to measure initial rates in the catechol-polyphenoloxidase system, the oxygen tension drops considerably during the first 2 min. Thus in systems containing ascorbic acid where the quinone condensation products are not formed until all the ascorbic acid has been oxidized, it is essential to bubble air through to ensure a constant oxygen tension in solution during the whole course of the reaction.

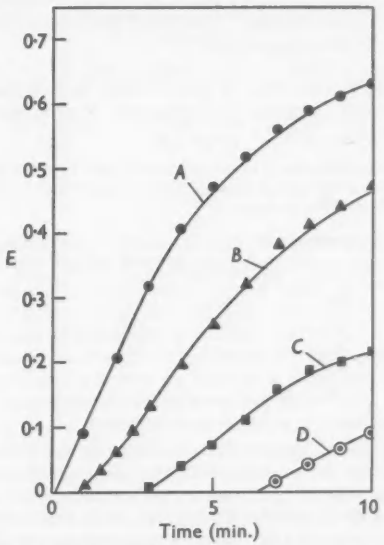

Fig. 5. Effect of increasing ascorbic acid concentration on the rate of enzyme action. *A*, no ascorbic acid added; *R* (graph) = 0·10 *E*/min. *B*, 0·4 mg. ascorbic acid added; *R* (graph) = 0·06 *E*/min. *C*, 0·8 mg. ascorbic acid added; *R* (graph) = 0·04 *E*/min. *D*, 1·2 mg. ascorbic acid added; *R* (graph) = 0·025 *E*/min. In each experiment 0·5 ml. enzyme, 2 mg. of catechol and ascorbic acid as shown in a total volume 4·0 ml. was used.

The effect of ascorbic acid on the catechol-polyphenoloxidase system

Colorimetric measurements. When ascorbic acid is present in the catechol-polyphenoloxidase system there is a delay in the onset of colour development until all the ascorbic acid has been oxidized. If the activity of the enzyme is not affected by the vitamin the rate of colour development should then be the same as if no ascorbic acid had been added. The results presented in Fig. 5 show that this is not the case, and that ascorbic acid in increasing concentration has a marked inhibitory effect on the initial rate of reaction. Amounts of ascorbic acid above 3 mg. prevented colour development completely.

Measurement by Sreerangachar's method. In order to demonstrate that the results obtained in the experiment described above were due to a loss in the oxidizing activity of the enzyme, a series of determinations were carried out using Sreerangachar's method, with increasing amounts of ascorbic acid and the same concentration of enzyme and catechol. It was argued that if ascorbic acid has no inhibitory effect, the same concentration of enzyme should be capable of oxidizing the same quantity of ascorbic acid irrespective of the concentration of the latter. The results in Table 2 show that this is not the case, and hence it must be concluded that ascorbic acid does inhibit the enzyme.

Table 2. *The effect of ascorbic acid on polyphenol-oxidase activity measured by Sreerangachar's method*

(Reaction volume 10 ml.; enzyme 0·5 ml., catechol 2 mg., with ascorbic acid as shown. These represent the average of duplicate determinations.)

Ascorbic acid (mg.)		Loss of ascorbic acid (mg.)	Per cent of fastest rate
At start	After 5 min.		
0·87	0·43	0·44	100
1·69	1·32	0·37	84
4·00	3·77	0·23	52
7·94	7·80	0·14	32

The effect of ascorbic acid oxidation products on the reaction

From the results obtained above it was not clear whether ascorbic acid itself or the oxidation products formed during the course of the reaction were the true inhibitors. In order to determine between these alternatives, the effect of dehydroascorbic acid and solutions of ascorbic acid oxidation products obtained by aerating a solution of ascorbic acid both in the presence and absence of added copper were tested. For the preparation of such solutions, air was drawn through a mixture of ascorbic acid and buffer for 2 hr. The results are shown in Tables 3 and 4.

It is apparent that in similar concentrations to those in which ascorbic acid shows a marked effect (about 0·4 mg.) neither dehydroascorbic acid nor the complicated oxidation products of ascorbic acid have much inhibitory action. In much higher concentrations, however, the oxidation products of ascorbic acid do affect the enzyme (Table 4). The smaller inhibition shown by the solution containing copper is explicable by the results shown later, where the effect of copper on the system is demonstrated.

It can be seen that the tube with no added copper (*A*) must have contained traces of a metal catalyst, since little ascorbic acid remained after 2 hr. (cf. Mapson, 1945), and this emphasizes the need for thorough precautions in determining the oxidation rates of polyphenoloxidase systems in the presence of ascorbic acid.

Table 3. *The effect of dehydroascorbic acid on the catechol-enzyme system*

(0·5 ml. enzyme + 2 mg. catechol in a total volume of 4·0 ml. in each experiment.)

Compound added	Amount (mg.)	Initial rate (*R* (graph))	Per cent maximum
None	—	0·16	100
Dehydroascorbic acid	0·34	0·16	100
Ascorbic acid	0·40	0·05	31

Table 4. *The effect of ascorbic acid oxidation products on the catechol-enzyme system*

(0·5 ml. enzyme, 2 mg. catechol plus additions as shown in a total vol. of 4·0 ml. in each experiment. Soln. *A* contained originally 625 μg./ml. of ascorbic acid in buffer solution. Soln. *B* contained originally 625 μg./ml. of ascorbic acid and 5 μg./ml. of copper in buffer solution. Both solutions aerated in buffer for 2 hr. before testing.)

Solution added	Volume (ml.)	Total ascorbic acid present (μg.)	Initial rate (*R* (graph))	Per cent maximum
Nil	—	0	0·21	100
A	0·5	1·3	0·21	100
B	0·5	0	0·21	100
A	3·0	7·8	0·09	43
B	3·0	0	0·14	67
Ascorbic acid	0·5	400	0·08	38

Correlation of the inhibitory activity with a direct effect of ascorbic acid on the enzyme

The effect of ascorbic acid on the substrate. Although it was clear from the foregoing experiments that, unless very high concentrations were used, ascorbic acid and not its oxidation products is involved in the inhibition of the enzyme-catechol system, there was a possibility that it reacted with the substrate and prevented the formation of brown condensation

Table 5. *Effect of added enzyme or substrate on the catechol-oxidized ascorbic-acid-enzyme system*

(Originally, 0·5 ml. enzyme, 2 mg. catechol + ascorbic acid as shown in total volume 4·0 ml. in each experiment. Additions made after 10 min.)

Addition after all ascorbic acid had disappeared	Ascorbic acid originally present (mg.)	Initial rate (*R* (graph))	Per cent maximum
None	0	0·125	100
None	1	0·05	40
Catechol (2 mg.; 0·5 ml.)	1	0·055*	44
Enzyme (0·5 ml.)	1	0·105*	84

* Corrected for volume increase.

products. However, when all the ascorbic acid had just disappeared (10 min.) from an ascorbic-acid-enzyme-catechol system only the addition of extra enzyme, and not extra catechol, increased the activity. The results are shown in Table 5.

It is evident that if the ascorbic acid reacted with the substrate or its oxidation products to prevent the formation of coloured compounds, addition of extra catechol after all the ascorbic acid had disappeared would increase the rate to that of the control, whereas addition of extra enzyme to such a system would have little or no effect. Since the reverse is the case, ascorbic acid must act on the enzyme itself.

The effect of ascorbic acid on the enzyme. That the conclusions reached in the previous experiment were correct was demonstrated in the following way.

Polyphenoloxidase solution (2 ml.) and varying amounts of ascorbic acid were diluted to constant volume (10 ml.) with buffer and oxygen-free nitrogen passed through the solutions at a slow rate (about 100 ml./hr.) for 2 hr. at room temperature. The solutions were then dialysed overnight against running (copper-free) water until completely free of ascorbic acid and made up to volume. One ml. of each solution was diluted with buffer (2·5 ml.) and its activity against catechol (2 mg.: 0·5 ml.) tested in the usual way. The results are shown in Table 6.

Table 6. *Effect of ascorbic acid on polyphenoloxidase under anaerobic conditions*

(Enzyme solution (2·0 ml.) in total volume of 10 ml. treated with varying amounts of ascorbic acid as shown under N_2 for 2 hr. After removal of excess ascorbic acid by dialysis, 1 ml. of the enzyme solution was taken with 2 mg. catechol in total volume 4·0 ml.)

Ascorbic acid added initially (mg.)	pH of initial solution	Ascorbic acid destroyed after 2 hr. under N_2 (% initial)	Initial rate (R (graph))	Per cent maximum
0	5·98	0	0·075	100
4	5·94	5	0·055	73
8	5·94	3	0·045	60
12	5·93	0	0·03	40

More reactive enzymes were naturally less affected, but it is immediately apparent from the results that ascorbic acid, since little or none is lost during the period of contact under nitrogen, itself inhibits the enzyme. It must be stressed that in the activity measurements shown in Table 6 no ascorbic acid is present and there is no delay in the onset of colour development.

The effect of copper on the system

In the absence of ascorbic acid. We have found with our enzyme system that the addition of extra cupric ion greatly increases the measurable activity, although catechol itself is not affected by copper in the absence of the enzyme (Table 7).

Kertesz (1952) has recently shown that when polyphenoloxidases are prepared by extraction with potassium cyanide solution, the resulting copper-free protein (apoenzyme) may be reactivated by the addition of cupric ion. He found that the activity of his preparation against dopa was unaffected by the presence of excess copper, but with tyrosine, increasing amounts of copper gave increased rates of oxygen uptake and suggested that cupric ion catalysed the non-enzymic oxidation of tyrosine by traces of *o*-quinone present.

Table 7. *Influence of copper on polyphenoloxidase activity*

(Enzyme 0·5 ml., catechol 2 mg. and Cu^{2+} as shown. Total vol. 4·0 ml.)

Cu^{2+} added (μg.)	Initial rate (R (graph))	Per cent control
0	0·11	100
25·5	0·20	182
51	0·32	290

Polonovski & Gonnard (1951) found that addition of cupric ion to polyphenoloxidase-dopa systems increased the production of secondary coloured quinone compounds but inhibited the formation of melanin. It has been shown that cupric ion alone increases the rate of oxidation of several phenolic compounds as shown by the colour produced and the oxygen uptake (Bhagvat & Richter, 1938).

It is likely, therefore, that the observed acceleration with added copper in our system is mainly due to the effect on the formation of coloured condensation products from the *o*-benzoquinone and not to a direct increase in the enzyme activity as such.

In the presence of ascorbic acid. As was expected from the results presented above, the presence of copper had a marked effect on enzyme systems containing ascorbic acid. Kertesz (1951) has shown that for his system of apoenzyme (copper-free), cupric ion and catechol, the oxidation of ascorbic acid is dependent on two different mechanisms: (i) oxidation by free cupric ion (Barron, de Meio & Klemperer, 1936), and (ii) oxidation by the protein-bound copper in the presence of catechol (Kubowitz, 1938).

The addition of excess cupric ion to our system involves mainly mechanism (i) and, as can be seen from the Table 8, there is a large decrease in the lag period for the onset of browning, indicating that ascorbic acid is oxidized rapidly. It was then expected that the rates obtained when all the ascorbic acid had been oxidized would be comparable with those obtained in its absence (Table 7). The initial rates are, however, much lower, although it is apparent from Fig. 6 that there is an autocatalytic effect after 1–1·5 min. This suggests that the unbound Cu^{+} produced during the oxidation of

ascorbic acid by mechanism (i) does not activate the system to the same extent as the cupric ion but, when re-oxidized by the air stream, exerts its effect producing the 'autocatalytic' appearance of the curves in Fig. 6. There is also the possibility that some copper may be lost by chelation with the oxidation products (cf. Mapson, 1941).

Table 8. *Influence of copper on the ascorbic-acid-enzyme systems*

(0·5 ml. enzyme, 2 mg. catechol and additions of ascorbic acid and of Cu^{2+} as shown. Total volume 4·0 ml.)

Cu^{2+} added (μg.)	Ascorbic acid added (μg.)	Delay period before onset of colour (min.)	Initial rate (R (graph))	Per cent control
0	0	0	0·11	100
0	400	0·9	0·095	86
51	400	0·2	0·145	127
0	800	5·7	0·055	50
51	800	1·3	0·10	91

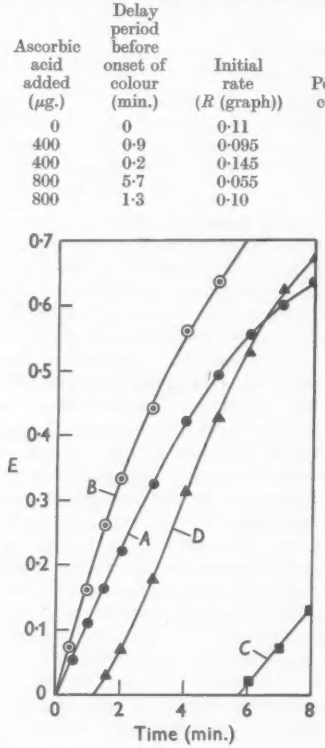

Fig. 6. Effect of Cu^{2+} on the polyphenoloxidase-catechol system, with and without ascorbic acid. *A*, no addition; *B*, 51 μg. Cu^{2+} added; *C*, 0·8 mg. ascorbic acid added; *D*, 51 μg. Cu^{2+} and 0·8 mg. ascorbic acid added. 0·5 ml. enzyme, 2 mg. catechol and additions as shown. Total volume 4·0 ml. in each case.

Influence of copper on the ascorbic-acid-'inactivated' enzyme. It has already been shown that the polyphenoloxidase is inactivated by ascorbic acid. It was thought that this might well have been due to the effect of the ascorbic acid on the enzymically-bound copper, and that addition of extra copper to the system would reverse the inhibition. Consequently, excess copper (51 μg. as $CuSO_4$) was added to enzyme solutions which had been treated as in Table 6 and their activity measured in the usual way. The results are shown in Table 9.

Table 9. *Influence of copper on the inactivated enzymes*

(Enzyme solution (2·0 ml.) in a total volume of 10 ml. treated with varying amounts of ascorbic acid as shown under N_2 for 2 hr. After removal of excess ascorbic acid by dialysis 1 ml. of the enzyme solution was taken with 2 mg. of catechol and Cu^{2+} as shown. Total volume 4·0 ml.)

Ascorbic acid added initially (mg.)	Cu^{2+} present (μg.)	Initial rate (R (graph))	Per cent control	Per cent initial rate with added Cu^{2+}
0	0	0·035	100	100
0	35	0·06	172	172
8	0	0·02	57	100
8	35	0·035	100	175
16	0	0·013	37	100
16	35	0·025	71	192

It can be seen that although the addition of excess copper does indeed bring the rate back to that of the untreated control, the increase in rate is roughly the same for each enzyme used (about 75%). Kertesz (1952) has shown that extremely small amounts of copper (<5 μg.) are required to reactivate his apoenzyme completely. Hence if the enzymically-bound copper had merely been removed during the treatment with ascorbic acid, the addition of the great excess of copper ion would have been expected to increase the observed activity of the inactivated enzyme to a much greater extent than the untreated control, since the excess copper, over the amount required for enzyme reactivation, would then increase the observed activity as shown in Table 7. Thus it appears that the active centres of the protein moiety of the enzyme have been irreversibly affected by the treatment with ascorbic acid.

DISCUSSION

From the results obtained in our experiments there can be no doubt that ascorbic acid exerts a considerable inhibitory effect on potato polyphenoloxidase which is not completely reversed when copper is added to the ascorbic-acid-free system.

It is apparent, therefore, that if the enzyme activity is measured in the presence of ascorbic acid by the chronometric method, by Sreerangachar's method (Asimov & Dawson, 1950) or manometrically (Krueger, 1950), erroneous results may be obtained even when precautions are taken to exclude ionic copper from the solution.

Krueger's results may be partially explained on the assumption that he used ordinary distilled water for his experiments (Kertesz (1951) reported that his 'ordinary' distilled water contained 0·4 μg. copper/ml.). The 'pro-oxidant' effect of ascorbic acid on the system which he observed, could then be due to oxidation of ascorbic acid by ionic copper without the intervention of the enzyme at all. His observations on the decreased rate of formation of dopa from tyrosine in the presence of ascorbic acid are in accord with our results.

Asimov & Dawson (1950), working on the inactivation of mushroom polyphenoloxidase during the oxidation of catechol, stated that 'the possibility of a "slow down" effect due to ascorbic acid itself, or to its degradation products, would also appear to be non-existent, since the course of oxygen uptake (as measured manometrically) has been shown, in these laboratories, to be unaffected by the presence or absence of ascorbic acid.' They measured enzyme activity both by the chronometric method and by Sreerangachar's method and, in view of our results, it is not surprising that they could find no simple equation to fit their inactivation rates.

It appears likely therefore that in much recent work on the nature of polyphenoloxidase systems, in which measurements have been made in the presence of ascorbic acid, the role of ionic copper has been insufficiently considered. The effect of ascorbic acid on polyphenoloxidase may be different *in vivo* and we hope to investigate this point later.

SUMMARY

1. The effect of ascorbic acid and its oxidation products on the activity of potato polyphenoloxidase has been studied using a modified colorimetric procedure.

2. It is shown that the enzyme undergoes a marked denaturation in the presence of ascorbic acid which is not reversed by ionic copper.

3. It is deduced that copper ions catalyse the rate of formation of coloured condensation products from *o*-benzoquinone.

4. Previous work on polyphenoloxidase systems is discussed in the light of our findings.

The authors wish to thank Dr L. W. Mapson for helpful suggestions and advice. Mr D. Wardale carried out the determination of dehydroascorbic acid and Mr C. Bignell constructed the apparatus. We would also like to record our appreciation of the encouragement given by Dr E. C. Bate-Smith, and one of us (P.B.) thanks the Government of Assam for financial support.

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Further Studies on the Properties and Assay of Glucose 6-Phosphate Dehydrogenase and 6-Phosphogluconate Dehydrogenase of Rat Liver

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Renewed interest in the direct oxidative pathway of glucose 6-phosphate metabolism during the last few years has revealed that this pathway is by no means restricted to erythrocytes, yeast and micro-organisms. The triphosphopyridine-nucleotide-(TPN)-specific glucose 6-phosphate and 6-phosphogluconate dehydrogenases are also widely distributed in mammalian tissues (Dickens & Glock, 1950, 1951; Horecker & Smyrniotis, 1951), in a variety of lower plants and animals (Cohen, 1950) and also in higher plants (Conn & Vennesland, 1951; Gibbs, 1952). The significance of this pathway in animal tissues, its physiological control and the relative importance of the direct oxidative and glycolytic routes of carbohydrate metabolism are still, however, chiefly matters of conjecture. An essential preliminary step to such an investigation is to devise a satisfactory procedure for the assay of glucose 6-phosphate and 6-phosphogluconate dehydrogenases in animal tissues and it was with this object in view that the present work was undertaken.

EXPERIMENTAL

Materials

D-*Glucose* 6-*phosphate* (G 6-P). This was prepared as the heptahydrate of the barium salt. Embden ester was dissolved in water, seeded with a few crystals of the heptahydrate, kindly given by Dr B. L. Horecker, adjusted to pH 8·0 with saturated $Ba(OH)_2$ solution and left overnight at 4°. The insoluble heptahydrate (Horecker, 1952) which crystallized out was washed with ethanol and acetone. It was 99·5% pure (analysed with Zwischenferment according to Kornberg, 1950).

Fructose 6-*phosphate* (F6-P). This was a preparation of the barium salt free from G 6-P.

6-*Phosphogluconate* (6-PG). A preparation of the barium salt of 6-phosphohexonate was used as a source of 6-PG in most of the experiments. Enzymic assay with a partially purified liver 6-PG dehydrogenase (see Methods section) showed that it contained 77% 6-PG. For P and sugar analysis see Dickens & Glock (1951). In later experiments a preparation of 6-PG prepared from the heptahydrate of G 6-P (barium salt) according to Robison & King (1931) was used. (Found: 6-PG, 87 (by enzymic assay); G 6-P, 0·0 (by enzymic assay); organic P, 6·4; inorganic P, 0·0%. Calc.: P, 6·47%.) Both phosphohexonate and fructose 6-phosphate were kindly provided by Dr Marjorie Macfarlane from the collection of the late Prof. R. Robison.

Ribulose 5-*phosphate*. An equilibrium mixture of the barium salts of ribulose 5-P and ribose 5-P (containing respectively 65 and 35% of these two esters) was used. We are indebted to Dr B. L. Horecker for this preparation.

D-*Ribose* 5-*phosphate* (R 5-P) was prepared from barium inosinate according to Marmur, Schlenk & Overland (1951). (Found: organic P, 8·41; inorganic P, 0·0; pentose (orcinol reaction), 41·2%. Calc.: P, 8·48; pentose, 41·1%.)

Triphosphopyridine nucleotide (TPN). This was made from horse liver by the unpublished method of Kornberg and Horecker (Horecker, 1952), involving purification of a crude preparation, obtained by Hg precipitation, on Dowex 1 formate. It contained 70% TPN (analysed with Zwischenferment according to Kornberg, 1950) and no DPN (by the alcohol-dehydrogenase method of Racker, 1950).

Cystine, o-*iodosobenzoate and* p-*chloromercuribenzoate*. These were prepared by Mr W. Lawson, Miss D. Salmony and Mr M. Woodford, respectively, to whom we are indebted.

Glutathione. This was presented by Distillers Co. Ltd.

Methods

Preparation of liver supernatants and differential centrifugation for studying intracellular distribution of dehydrogenases. Livers from rats which had previously fasted overnight were chilled to 0° and disintegrated in a Potter glass homogenizer in 8 vol. of ice-cold isotonic (0·15M) KCl containing $KHCO_3$ (8 ml. 0·02M-$KHCO_3$/l.) to maintain the pH at 7·0 (LePage, 1948). When the supernatant only was required, for the assay of the dehydrogenases, the suspension was centrifuged at 4000 *g* for 60 min. at 2–4° in an International refrigerated centrifuge (Type PR 1) and the supernatant ('supernatant I') siphoned off and dialysed overnight against the same extracting medium at 2°. For determining the intracellular distribution of the dehydrogenases, the suspension was divided into three parts, one part ('suspension') kept, one part centrifuged as above ('supernatant I') and the third part centrifuged at 20000 *g* for 60 min. at 2–4° ('supernatant II'). Supernatants I and II were then diluted with the isotonic extracting medium up to the original volume of the suspension used and all three parts dialysed as described above. Except where otherwise stated, reference to 'supernatant' in the text refers to 'supernatant I'.

Preparation of 6-*phosphogluconate dehydrogenase from liver supernatants*. The fraction, precipitating between 0·6 and 0·7 saturation with $(NH_4)_2SO_4$ at pH 7·3 from pooled liver supernatants at 4°, was collected by centrifuging at 4° and dialysed overnight against distilled water at this temperature. It was diluted to one-tenth of the original volume of the supernatant and stored at −15°. The protein

content, determined by the biuret method of Robinson & Hogden (1940), was 10·7 mg. protein/ml. This fraction contained a very active 6-PG dehydrogenase (approximately 10 times as active as the original liver supernatant) and no G 6-P dehydrogenase. It was used in the assay of G 6-P dehydrogenase (see below).

Estimation of glucose 6-phosphate and 6-phosphogluconate dehydrogenase activities. Dehydrogenase activity was determined spectrophotometrically by following the rate of reduction of TPN at 340 mμ. in 1 cm. cells at room temperature. In the final assay procedure, the reaction mixture consisted of 0·05–0·10 ml. liver supernatant (1·3–1·6 mg. N/ml.), 0·5 ml. 0·1 M-$MgCl_2$, 0·5 ml. 0·25 M-glycylglycine buffer (pH 7·6 for G 6-P dehydrogenase and pH 9·0 for 6-PG dehydrogenase) and 0·2 mg. TPN in a total volume of 2·4 ml. The reaction was started by the addition of 0·1 ml. 0·05 M substrate to both cells, the blank cell being devoid of TPN.

The activity measured at pH 7·6 with G 6-P as substrate is the summation of G 6-P and 6-PG dehydrogenase activities. In order to determine G 6-P dehydrogenase activity only, two alternative procedures were adopted. In one method, dehydrogenase activity of liver supernatants was determined in the presence of a large excess of 6-PG dehydrogenase (0·1 ml. dialysed $(NH_4)_2SO_4$ fraction described above). Under these conditions, 50 % of the total activity is due to G 6-P dehydrogenase. In the second method, the G 6-P dehydrogenase activity was taken as the difference between the activities measured separately at pH 7·6 in the presence of 0·1 ml. 0·05 M-6-PG and 0·1 ml. of a solution containing G 6-P and 6-PG (0·05 M in respect to each).

The increase in density at 340 mμ. during the first 5 min. was taken as a measure of dehydrogenase activity. All the activities are expressed at 20° by applying an experimentally determined temperature coefficient of 1·7 (for the 10° temperature range between 20 and 30°) for both dehydrogenases.

A unit of enzyme activity is defined as the quantity of enzyme which, at 20° and the given pH, reduces 0·01 μmole TPN/min., based on the readings over the first 5 min.

With the whole suspension, it was not possible to measure dehydrogenase activity directly in the spectrophotometer cells on account of the opacity of the reaction mixtures, and for this reason the reaction was allowed to proceed at room temperature, and at 1 min. intervals 2·5 ml. samples were removed and introduced into stoppered centrifuge tubes containing 2·5 ml. ethanol, and 0·1 ml. 10 % (w/v) Na_2SO_4 solution added to aid flocculation. After 30 min. at room temperature to allow for complete precipitation of the protein, the tubes were spun for 5 min. and the clear supernatant used for spectrophotometric assay of reduced TPN. The suitability of this ethanol-precipitation procedure was tested on liver supernatants, and identical results were obtained for the activity of both dehydrogenases by direct and precipitation procedures.

In most of the experiments when the action of metal ions was being studied, dehydrogenase activities were measured at pH 8·4 in a final concentration of 0·01 M-tris(hydroxymethyl)aminomethane (Tris) buffer.

Isomerase. Isomerase activity was determined by substituting F 6-P for G 6-P.

Phosphatase. Phosphatase activity towards G 6-P and 6-PG was tested under the conditions of the G 6-P and 6-PG dehydrogenase assay procedure previously described, by determining the liberation of inorganic phosphate.

Enzymic breakdown of TPN including conversion to diphosphopyridine nucleotide (DPN). Enzymic breakdown of TPN resulting in the formation of free nicotinamide was determined in the whole suspension and in supernatants I and II at pH 7·6 under the conditions of the dehydrogenase assay procedure. Protein and unchanged TPN were precipitated with $Zn(OH)_2$ (McIlwain & Rodnight, 1949) and any liberated nicotinamide determined colorimetrically by the procedure of Melnick & Field (1940). Conversion of TPN to DPN by liver supernatants was determined by allowing 0·1 ml. liver supernatant to react with 0·2 mg. TPN at room temperature for 30 min. and then determining DPN with alcohol dehydrogenase (Racker, 1950).

Action of compounds reacting with sulphydryl groups. Iodoacetamide, *o*-iod osobenzoate, *p*-chloromercuribenzoate, *p*-aminophenylarsenoxide and cystine were used as inhibitors and cysteine and glutathione as reactivators. All experiments were carried out anaerobically in N_2 at 30° in Warburg manometers, the liver supernatant (1 ml.), neutralized inhibitor (0·1 ml.) and veronal buffer, pH 7·3, being introduced into the main chamber and neutralized reactivator (0·1 ml.) into the side arm, the total volume in each case being 2·0 ml. The inhibitors were allowed to react with the enzyme for 10 min. (in the case of *p*-chloromercuribenzoate) or 30 min. (with the other inhibitors) before the reactivator was added and incubation then continued for a further 30 min. After cooling rapidly to room temperature G 6-P and 6-PG dehydrogenase activities were measured in veronal buffer at pH's 8·5 and 9·8, respectively. G 6-P dehydrogenase activity was measured by the method employing two substrates. Suitable incubated controls were included.

Competitive inhibition of dehydrogenase by adenosine triphosphate (ATP). The G 6-P and 6-PG dehydrogenase activities of liver supernatants were determined as in the assay procedure, but with four different levels of TPN (0·05, 0·07, 0·1 and 0·2 mg.) and, at each level of TPN, without ATP and with the addition of 0·2 ml. or 0·5 ml. 0·06 M-ATP.

Nitrogen contents of liver fractions. These were determined by the micro-Kjeldahl procedure.

RESULTS

The direct oxidative pathway of G 6-P metabolism proceeds in a cycle via 6-PG, ribulose phosphate and probably sedoheptulose phosphate (Horecker, Smyrniotis & Seegmiller, 1951; Horecker & Smyrniotis, 1952) back to hexosemonophosphate (see Dische, 1951). Of these various stages only the oxidation of G 6-P and the oxidative decarboxylation of 6-PG are TPN-dependent. It thus follows that when the rate of reduction of TPN is taken as a measure of dehydrogenase activity, the activity determined with G 6-P as substrate represents a summation of G 6-P and 6-PG dehydrogenase activities. Moreover, the assay of G 6-P and 6-PG dehydrogenases by such a method is only valid if there is no detectable formation of G 6-P from 6-PG. To establish this point, an equilibrium mixture of ribulose 5-phosphate and R 5-P was used as substrate and reduction of TPN measured under the conditions of the assay procedures for G 6-P and 6-PG dehydrogenases, in glycylglycine at pH's 7·6

and 9·0 respectively. In neither case was there any reduction of TPN, thus indicating that no G 6-P was formed (Fig. 1). These results do not necessarily mean that the complete system for resynthesis of G 6-P from ribulose phosphate is not present in the liver supernatants used for the assay of G 6-P and 6-PG dehydrogenases, but rather that the conditions are not optimal particularly in respect to

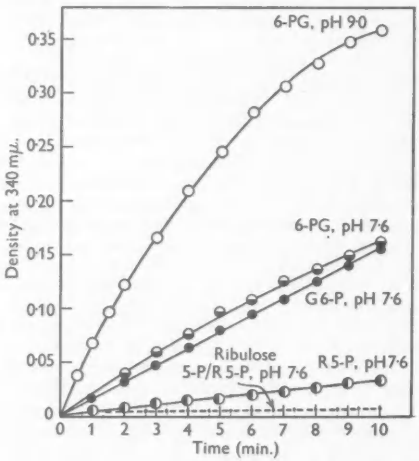

Fig. 1. Oxidation of 6-phosphogluconate, glucose 6-phosphate, ribose 5-phosphate and ribulose 5-phosphate by rat-liver supernatant. Rate of reduction of TPN measured at 340 mμ. in 0·05 M-glycylglycine buffer, at pH 7·6 or 9·0, in the presence of 0·1 ml. liver supernatant, 0·5 ml. 0·1 M-$MgCl_2$, 0·2 mg. TPN and 0·1 ml. substrate (0·05 M in respect to 6-PG, G 6-P and R 5-P and 0·001 M in respect to an equilibrium mixture of ribulose 5-P/R 5-P). Concentration of ribulose 5-P used approximately equivalent to the G 6-P oxidized under the conditions of the assay procedure. Total volume, 2·5 ml. ○—○, 6-PG, pH 9·0; ◒—◒, 6-PG, pH 7·6; ●—●, G 6-P, pH 7·6; ◐—◐, R 5-P, pH 7·6; – – · – – ·, ribulose 5-P/R 5-P, pH 7·6.

temperature, time of reaction and substrate concentration. Fig. 1 does indeed show that with a large excess of R 5-P there is some reduction of TPN at room temperature, although with the concentration of R 5-P which could be formed from 6-PG under the conditions of the assay of 6-PG dehydrogenase, this is negligible.

In order to determine G 6-P dehydrogenase activity as distinct from the summation of G 6-P and 6-PG dehydrogenases, two alternative procedures have been adopted. In one method, dehydrogenase activity of liver supernatants was determined in glycylglycine buffer at pH 7·6 with G 6-P as substrate in the presence of a large excess of 6-PG dehydrogenase. Under these conditions the true G 6-P dehydrogenase activity is 50 % of the total measured activity. In the second method, the G 6-P dehydrogenase activity was taken as the difference between the activities measured at pH 7·6 with 6-PG only and with both substrates together. There was good agreement between the results obtained by these two methods. The mean values for G 6-P dehydrogenase activity in units/g. liver (1 unit being defined as the quantity of enzyme which reduces 0·01 μmole TPN/min. at 20°) for a group of six female rats, together with the standard errors, were 98 ± 15 by the method using two substrates and 96 ± 14 by the method with excess 6-PG dehydrogenase. The corresponding uncorrected value for G 6-P dehydrogenase activity was 160 ± 21, indicating that, at least in the livers of normal rats, approximately 60 % of the dehydrogenase activity measured at pH 7·6 with G 6-P as substrate is due to G 6-P dehydrogenase and the remaining 40 % to 6-PG dehydrogenase.

For routine estimation of G 6-P dehydrogenase activity the method using excess 6-PG dehydrogenase is preferable, since only one measurement has to be made and the reaction rate is linear. The method employing two substrates, however, was found to have special application in connexion with the inhibition by heavy metals and sulphydryl compounds.

pH-*Activity curves of glucose 6-phosphate and 6-phosphogluconate dehydrogenases*

The pH-activity curves for both dehydrogenases in 0·05 M glycylglycine and 0·006 M veronal, using liver supernatants as the source of the dehydrogenases, are given in Figs. 2 and 3. The pH optimum for 6-PG dehydrogenase is consistently higher than for G 6-P dehydrogenase. In glycylglycine buffer, the pH optima are 9·0 for 6-PG dehydrogenase and 7·6 for G 6-P dehydrogenase, although in the latter case the activity falls off only slightly on the alkaline side of the optimum. In veronal buffer, the corresponding pH optima are at 9·8 and 8·5 respectively. The pH optima in glycylglycine were unaffected by the addition of magnesium chloride in a final concentration of 0·02 M or in the case of the G 6-P dehydrogenase by the addition of excess 6-PG dehydrogenase.

The final assay procedure was carried out in glycylglycine in order to remove traces of inhibitory heavy metals. The high pH optimum for 6-PG dehydrogenase is in contrast to the value of 7·6 found for purified yeast 6-PG dehydrogenase by Horecker & Smyrniotis (1951).

The effect of metal ions on dehydrogenase activities

The activation of both dehydrogenases by Mg^{2+} and Ca^{2+} in glycylglycine is shown in Fig. 4. Although the degree of activation is very variable with different enzyme preparations, the optimal

concentration of Mg^{2+} and Ca^{2+} for both dehydrogenases is approximately 2×10^{-2}M. Magnesium in this final concentration was used as the activating ion in the assay procedure. Cohen (1951) also found

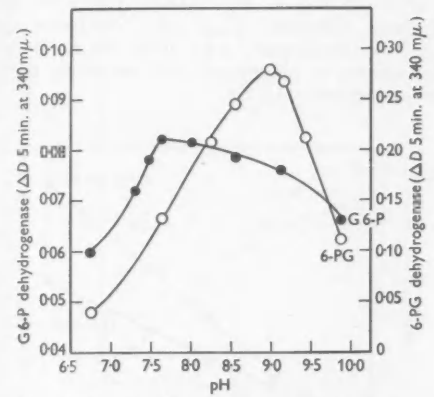

Fig. 2. pH-Activity curves of glucose 6-phosphate and 6-phosphogluconate dehydrogenases in glycylglycine buffer. Dehydrogenase activities determined as described in the Methods section with 0·1 ml. liver supernatant, without the addition of Mg^{2+}. ●—●, G 6-P dehydrogenase; ○—○, 6-PG dehydrogenase.

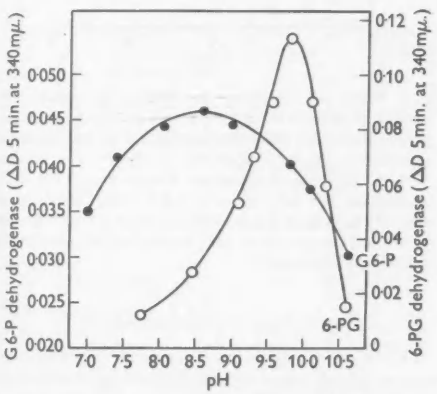

Fig. 3. pH-Activity curves of glucose 6-phosphate and 6-phosphogluconate dehydrogenases in veronal buffer. Dehydrogenase activities determined with 0·1 ml. liver supernatant in 0·006M veronal (veronal acetate/HCl or veronal/Na_2CO_3/HCl). ●—●, G 6-P dehydrogenase; ○—○, 6-PG dehydrogenase.

activation of both dehydrogenases of *Chaetopterus* by Mg^{2+} and Ca^{2+}, the optimal concentration of both ions for both dehydrogenases being at a final concentration of 3×10^{-2}M. With purified yeast 6-PG dehydrogenase, Horecker & Smyrniotis (1951) found that a concentration of 5×10^{-3}M Mg^{2+} produced maximal activation.

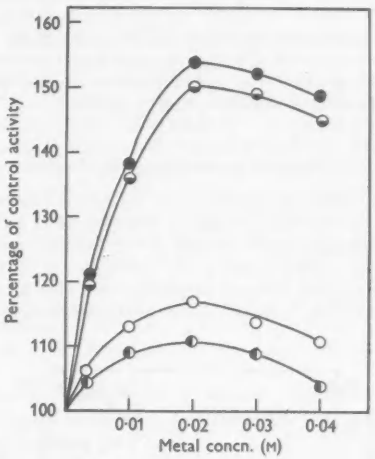

Fig. 4. Activation of glucose 6-phosphate and 6-phosphogluconate dehydrogenases by Ca^{2+} and Mg^{2+}. Dehydrogenase activities determined in glycylglycine buffer with 0·1 ml. liver supernatant as described in the Methods section. G 6-P dehydrogenase determined with excess 6-PG dehydrogenase. ●—●, G 6-P dehydrogenase, Mg^{2+}; ◒—◒, G 6-P dehydrogenase, Ca^{2+}; ○—○, 6-PG dehydrogenase, Mg^{2+}; ◐—◐, 6-PG dehydrogenase, Ca^{2+}.

Table 1. *Influence of metals on glucose 6-phosphate and 6-phosphogluconate dehydrogenase activities*

Metal ion	Final metal concn. (M)	Dehydrogenase activity as percentage of control*	
		G 6-P	6-PG
K^+	1×10^{-2}	117	101
Mg^{2+}	1×10^{-2}	152	120
Ca^{2+}	1×10^{-2}	154	120
Mn^{2+}	1×10^{-2}	152	128
Co^{2+}	1×10^{-4}	94	106
Al^{3+}	1×10^{-4}	109	104
Fe^{3+}	1×10^{-4}	93	91
Fe^{2+}	1×10^{-4}	100	94
Zn^{2+}	1×10^{-4}	82	3
Cu^{2+}	1×10^{-4}	103	18
Hg^{2+}	1×10^{-4}	0	0

* Dehydrogenase activity determined as described in the Methods section with 0·1 ml. liver supernatant in Tris buffer pH 8·4 (final concentration, 0·01M) G 6-P dehydrogenase activity determined by the method employing two substrates.

The action of other metal ions is summarized in Table 1. Manganese produced marked activation of both dehydrogenases and in this respect was at least as active as Mg^{2+}. Al^{3+}, Co^{2+}, Fe^{2+}, and Fe^{3+} in a final concentration of 1×10^{-4}M were almost

ineffective. Hg^{2+}, in a final concentration of 1×10^{-4} M, inhibited both dehydrogenases completely. 6-PG dehydrogenase was markedly inhibited by Zn^{2+} and Cu^{2+} at this concentration.

The previous findings of Dickens (1938) and Dickens & Glock (1951) that cyanide activates both dehydrogenases can probably be accounted for by the removal of inhibitory heavy metals.

Variations in substrate concentration

The effect of variations in substrate concentration on the initial rates of reaction of G 6-P dehydrogenase at pH 7·6 and of 6-PG dehydrogenase both at pH's 7·6 and 9·0 is shown in Fig. 5, these results being plotted according to Lineweaver & Burk (1934). The Michaelis constant for the G 6-P

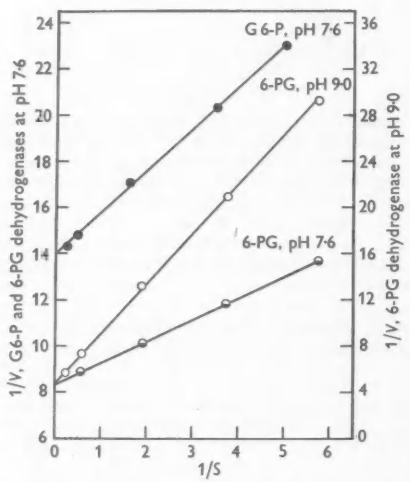

Fig. 5. Effect of variations in substrate concentration on glucose 6-phosphate and 6-phosphogluconate dehydrogenase activities. Dehydrogenase activities determined in glycylglycine buffer, as described in the Methods section, with 0·1 ml. liver supernatant. Values for $1/S$ to be multiplied by 10^4. ○—○, 6-PG dehydrogenase at pH 9·0; ◒—◒, 6-PG dehydrogenase at pH 7·6; ●—●, G 6-P dehydrogenase at pH 7·6 (determined with excess 6-PG dehydrogenase).

dehydrogenase is $1{\cdot}3 \times 10^{-5}$ M and for 6-PG dehydrogenase 9×10^{-5} M at pH 9·0 and approximately 1×10^{-5} M at pH 7·6, indicating a much greater affinity of 6-PG for its dehydrogenase at the lower pH. The value of the constant for purified yeast 6-PG dehydrogenase at pH 7·6 is 5×10^{-5} M (Horecker & Smyrniotis, 1951).

Variations in TPN concentration

The effect of variations in TPN concentration on the initial reaction rates of both dehydrogenases is shown in Fig. 6. Approximate values for the Michaelis constant, K_{TPN}, are $1{\cdot}3 \times 10^{-5}$ M for G 6-P dehydrogenase, $2{\cdot}4 \times 10^{-5}$ M for 6-PG dehydrogenase at pH 9·0 and $2{\cdot}8 \times 10^{-5}$ M for 6-PG dehydrogenase at pH 7·6.

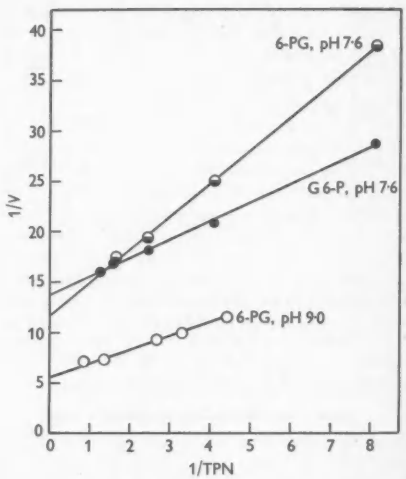

Fig. 6. Effect of variations in TPN concentration on glucose 6-phosphate and 6-phosphogluconate dehydrogenase activities. Dehydrogenase activities determined in glycylglycine buffer, as described in the Methods section, with 0·1 ml. liver supernatant. Values for 1/TPN to be multiplied by 10^4. ○—○, 6-PG dehydrogenase at pH 9·0; ◒—◒, 6-PG dehydrogenase at pH 7·6; ●—●, G 6-P dehydrogenase at pH 7·6 (determined with excess 6-PG dehydrogenase).

Variation in enzyme concentration

The effect of variations in enzyme concentration on the initial rates of reaction of both dehydrogenases is shown in Fig. 7. The reaction rate (rate of reduction of TPN) is directly proportional to enzyme concentration provided that not more than 40 % of the TPN is reduced during the first 5 min., which, under the conditions of the assay of both G 6-P and 6-PG dehydrogenases, amounts to the reduction of 0·08 μmole TPN. The range of proportionality can be extended by increasing the TPN concentration. In the case of G 6-P dehydrogenase, the rate of reaction is proportional to enzyme concentration over a slightly wider range using the method with excess 6-PG dehydrogenase than by the method

employing two substrates, owing to the fact that the total reduction of TPN is greater in the latter case.

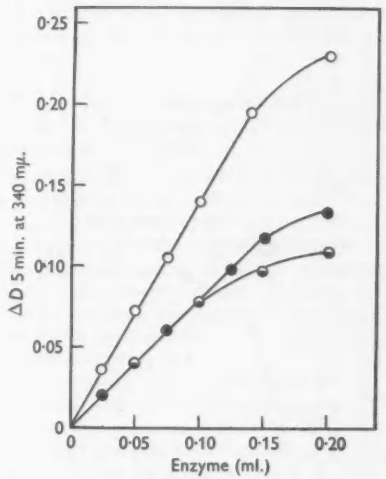

Fig. 7. Enzyme-concentration-activity curves for glucose 6-phosphate and 6-phosphogluconate dehydrogenases. Dehydrogenase activities determined in glycylglycine buffer with varying concentrations of liver supernatant (1·4 mg. N/ml.) as described in the Methods section. ○—○, 6-PG dehydrogenase; ●—●, G 6-P dehydrogenase (determined with excess 6-PG dehydrogenase); ◒—◒, G 6-P dehydrogenase (determined by method employing two substrates).

Table 2. *Levels of glucose 6-phosphate and 6-phosphogluconate dehydrogenases in rat liver*

	Δ_D at 340 mμ. in 5 min.* at 20°/ml. liver supernatant	Units/g. liver†
G 6-P dehydrogenase, pH 7·6		
Male rats (18)	0·64±0·04	46±3
Female rats (12)	1·44±0·16	104±12
6-PG dehydrogenase, pH 7·6		
Male rats (18)	0·82±0·11	59±8
Female rats (12)	1·80±0·16	130±12
6-PG dehydrogenase, pH 9·0		
Male rats (18)	2·03±0·14	147±10
Female rats (12)	3·99±0·22	290±16

* Δ_D of 1·0 corresponds to the reduction of 0·403 μmoles TPN.

† A unit of activity is defined as the amount of enzyme which reduces 0·01 μmole TPN/min. at 20°, based on the readings over the first 5 min. Results are expressed as means±S.E. of means.

Levels of glucose 6-phosphate and 6-phosphogluconate dehydrogenase activities in the liver of rat

Mean values for the G 6-P and 6-PG dehydrogenase activities in the livers of both male and female rats are given in Table 2. It is of interest that the levels of both enzymes are consistently higher in females than in males.

Intracellular distribution of dehydrogenases

Preliminary intracellular distribution experiments in isotonic sucrose indicated that all the G 6-P and 6-PG dehydrogenase activities were in the soluble fraction of disintegrated liver. Further distribution experiments were designed solely to confirm this finding. Isotonic potassium chloride was chosen as the suspending medium since cellular components are more readily agglutinated than in sucrose and lower speeds of centrifugation are required. The preparation of suspension, supernatant I (after spinning for 60 min. at 4000 *g*) and supernatant II (after spinning for 60 min. at 20 000 *g*) is described in the Methods section.

Table 3. *Distribution of glucose 6-phosphate and 6-phosphogluconate dehydrogenases in rat-liver suspensions*

(Method I, direct spectrophotometric estimation. Method II, ethanol-precipitation procedure. For experimental details and preparation of liver fractions see Methods section. Activity expressed as Δ_D/1 cm./340 mμ. in 5 min. at 20°.)

	G 6-P dehydrogenase activity		6-PG dehydrogenase activity	
Liver fraction	Method I	Method II	Method I	Method II
Suspension	—	0·148	—	0·142
Supernatant I	0·144	0·150	0·160	0·144
Supernatant II	0·146	0·140	0·146	0·140

Table 3 shows the results of a typical experiment, the dehydrogenase activities being determined both by the ethanol-precipitation procedure and by the direct spectrophotometric method. The results indicate that all three liver fractions have identical G 6-P and 6-PG dehydrogenase activities and that these dehydrogenases are therefore located exclusively in the soluble fraction. It is of interest that the glycolytic enzymes have the same intracellular distribution (LePage & Schneider, 1948).

Hydrolysis of substrates and TPN

Breakdown of substrates by phosphatases and of TPN by nucleotidase, as determined by the liberation of free nicotinamide, were negligible both in the suspensions and the supernatants when measured at room temperature under the conditions of the dehydrogenase assay. The dephosphorylation of TPN to DPN by liver supernatant was also very small, amounting to only 1 % conversion in 10 min. at room temperature.

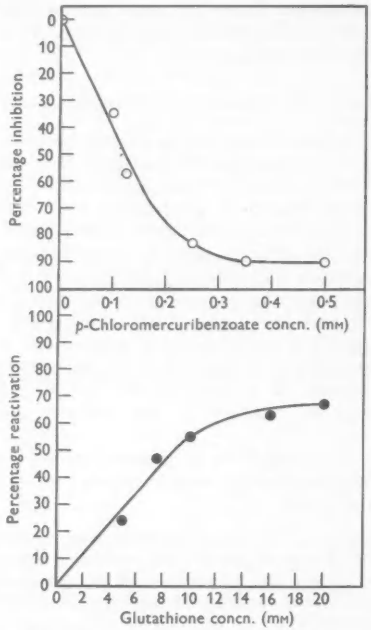

Fig. 8. Inhibition of 6-phosphogluconate dehydrogenase by *p*-chloromercuribenzoate and reversal by glutathione. Upper curve shows inhibition of 6-PG dehydrogenase by varying concentrations of *p*-chloromercuribenzoate and lower curve the reversal of the inhibition produced by $2{\cdot}5 \times 10^{-4}$ M mercurial with varying concentrations of glutathione. Incubations with mercurial allowed to proceed for 10 min. and in reactivation experiments followed by further incubation for 30 min. with glutathione. All incubations carried out in N_2 at 30° in veronal buffer, pH 7·3. 6-PG dehydrogenase activity determined in veronal-carbonate buffer at pH 9·8.

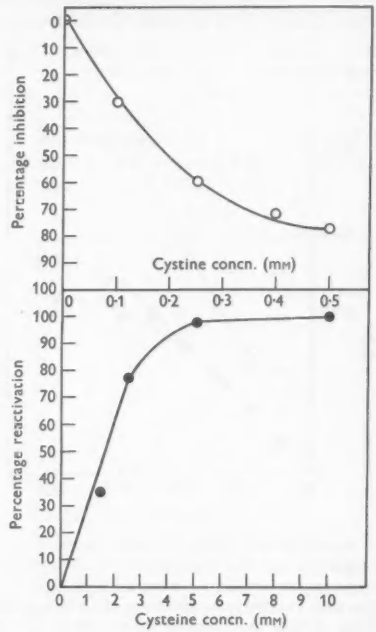

Fig. 9. Inhibition of 6-phosphogluconate dehydrogenase by cystine and reversal by cysteine. Upper curve shows inhibition of 6-PG dehydrogenase by varying concentrations of cystine and lower curve the reversal of the inhibition produced by $2{\cdot}5 \times 10^{-4}$ M-cystine with varying concentrations of cysteine. Incubations with cystine allowed to proceed for 30 min. and in reactivation experiments followed by further incubation for 30 min. with cysteine. All incubations carried out in N_2 at 30° in veronal buffer at pH 7·3. 6-PG dehydrogenase activity determined in veronal-carbonate buffer at pH 9·8.

Table 4. *Inhibition of glucose 6-phosphate and 6-phosphogluconate dehydrogenase activities by sulphydryl-combining compounds*

(For experimental details see Methods section. G 6-P dehydrogenase activity determined by method employing two substrates.)

	Inhibition of dehydrogenase activity (%)			Reactivation of dehydrogenase activity (%)	
Inhibitor	G 6-P	6-PG	Reactivator	G 6-P	6-PG
p-Chloromercuribenzoate, $2{\cdot}5 \times 10^{-4}$ M	91	83	Cysteine, $2{\cdot}5 \times 10^{-3}$ M	10	60
			Glutathione, 2×10^{-2} M	21	67
o-Iodosobenzoate, $2{\cdot}5 \times 10^{-4}$ M	62	76	Cysteine, $2{\cdot}5 \times 10^{-3}$ M	0	23
			Glutathione, 1×10^{-2} M	15	45
Cystine, $2{\cdot}5 \times 10^{-4}$ M	6	60	Cysteine, $2{\cdot}5 \times 10^{-3}$ M	0	78
p-Aminophenylarsenoxide, $2{\cdot}5 \times 10^{-4}$ M	0	0	—	—	—
Iodoacetamide, $2{\cdot}5 \times 10^{-3}$ M	12	16	—	—	—

Isomerase

Liver supernatants were found to have a very active isomerase since the dehydrogenase activities were identical whether F 6-P or G 6-P was used as substrate.

Effect of compounds reacting with sulphydryl groups on glucose 6-phosphate and 6-phosphogluconate dehydrogenase activities

The inhibition of both G 6-P and 6-PG dehydrogenase activities by low concentrations of Hg^{2+} and of 6-PG dehydrogenase by Cu^{2+} suggested that these enzymes might possess active SH groups. von Euler & Adler reported as early as 1935 that hexosemonophosphate dehydrogenase was inhibited by Cu^{2+} and bromoacetate. Horecker & Smyrniotis (1951) also observed marked inhibition of a purified yeast 6-PG dehydrogenase by Cu^{2+}.

In the present study, iodoacetamide, cystine, *o*-iodosobenzoate, *p*-chloromercuribenzoate and *p*-aminophenylarsenoxide were used as inhibitors and cysteine and glutathione as reactivators. Representative results are given in Table 4. Both dehydrogenases were inhibited by *p*-chloromercuribenzoate and *o*-iodosobenzoate, slightly greater inhibition being produced by the mercurial. The 6-PG dehydrogenase was, in addition, inhibited by cystine. Neither dehydrogenase was affected by the arsenical. Purified yeast G 6-P dehydrogenase (Kornberg, 1950) was also inhibited by the mercurial and by *o*-iodosobenzoate, but not by the arsenical.

Inhibition of 6-PG dehydrogenase by cystine, *p*-chloromercuribenzoate or *o*-iodosobenzoate was partially or completely reversed by cysteine and glutathione, but there was no striking reversal of G 6-P dehydrogenase inhibition by either reactivator. Inhibition of 6-PG dehydrogenase by *p*-chloromercuribenzoate and cystine and reversal by glutathione and cysteine, respectively, is shown in Figs. 8 and 9. Failure to obtain inhibition with arsenicals has also been reported for other enzymes, e.g. urease, which are known to contain active SH groups (Barron & Singer, 1945).

The results obtained with 6-PG dehydrogenase indicate that the activity of this enzyme is dependent on the presence of active sulphydryl groups, but it is uncertain whether the same applies to G 6-P dehydrogenase.

Competitive inhibition of 6-PG dehydrogenase by ATP

In a previous paper (Dickens & Glock, 1951) it was shown that nicotinamide acts as a competitive inhibitor of TPN utilization by 6-PG dehydrogenase but not of G 6-P dehydrogenase, and in addition that ATP caused some inhibition of 6-PG dehydrogenase activity. Feigelson, Williams & Elvehjem (1951) meanwhile found that nicotinamide behaved similarly in connexion with the DPN utilization by malic dehydrogenase, and this work was extended by Williams (1952) to include adenine, adenosine and ATP, which were all found to be more potent inhibitors than nicotinamide.

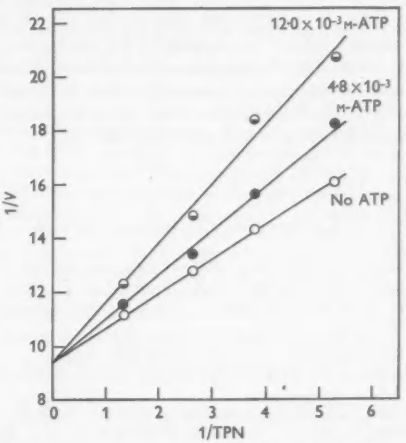

Fig. 10. Competitive inhibition of 6-phosphogluconate dehydrogenase by ATP. Activity determined as in Methods section with 0·1 ml. liver supernatant at four TPN concentrations. Positions of the lines calculated by the method of least squares. Values for 1/TPN to be multiplied by 10^4. ○—○, No ATP; ●—●, in the presence of 0·2 ml. 0·06 M ATP; ◒—◒, in the presence of 0·5 ml. 0·06 M ATP.

The action of ATP on the G 6-P and 6-PG dehydrogenase activities of liver supernatants has been studied and the results with 6-PG dehydrogenase, plotted according to Lineweaver & Burk (1934), are shown in Fig. 10. This indicates that ATP acts as a competitive inhibitor of 6-PG dehydrogenase since the slopes of the three curves (without ATP and with two different levels of ATP) are different but all cut the $1/V$ axis at the same intercept. These experiments were carried out at pH 9·0 as in the 6-PG dehydrogenase assay procedure. At pH 7·6 there was no evidence of competitive inhibition with G 6-P dehydrogenase. It would appear from these experiments that TPN is more firmly bound to G 6-P dehydrogenase than to 6-PG dehydrogenase.

SUMMARY

1. Both glucose 6-phosphate and 6-phosphogluconate dehydrogenases are present exclusively in the soluble fraction of disintegrated liver.

2. Conditions for the assay of these dehydrogenases in rat liver are described, and a unit of

activity defined as the quantity of enzyme which at 20° reduces 0·01 μmole triphosphopyridine nucleotide per minute.

3. The mean values of glucose 6-phosphate dehydrogenase activity, determined at pH 7·6, and of 6-phosphogluconate dehydrogenase activity, determined at pH's 7·6 and 9·0, were respectively 46 ± 3, 59 ± 8 and 147 ± 10 units/g. liver in male rats. All these levels were consistently higher in females.

4. Both dehydrogenases are activated by magnesium, calcium and manganese and inhibited by mercury. 6-Phosphogluconate dehydrogenase is also inhibited by cupric and zinc ions.

5. Evidence is presented that the activity of 6-phosphogluconate dehydrogenase is dependent on the presence of active sulphydryl groups.

6. Adenosine triphosphate acts as a competitive inhibitor of 6-phosphogluconate dehydrogenase but not of glucose 6-phosphate dehydrogenase.

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The Oxidation of Tryptophan in Pea-seedling Tissues and Extracts

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Tryptophan is known to be a source of nicotinic acid in animals (literature reviewed by Dalgliesh, 1951). There is evidence (Gustafson, 1949; Galston, 1949; Nason, 1950) that this conversion occurs also in some higher plants, but few of the intermediate compounds found in animals have been detected. One intermediate, anthranilic acid, accumulates in the anthers and seedling leaves of a maize mutant (Teas & Anderson, 1951).

Tryptophan may also be a precursor of indolylacetic acid in spinach leaves (Wildman, Ferri & Bonner, 1947), in tobacco ovaries (Wildman & Muir, 1949), in pineapple leaves (Gordon & Nieva, 1949) and in crown-gall tissue of sunflower (Henderson & Bonner, 1952). Conversion to indole, as by *Escherichia coli* (Happold, 1950) has not been reported in higher plants.

In the present work with pea seedlings, no conversion to indolylacetic acid was observed, but some experiments on oxidation of indolylacetic acid are reported. Certain similarities were found to the early stages of tryptophan breakdown in animal tissue.

Most studies of the mechanism of tryptophan oxidation have been made with cells of bacteria or animals previously exposed to an unusually high concentration of tryptophan (Stanier & Tsuchida, 1949; Knox & Mehler, 1950). In such cells adaptive enzymes were produced and tryptophan was

oxidized much faster than in normal cells. Of several plant tissues examined, pea seedlings contained the fastest tryptophan-oxidizing system. Seedlings of maize and dwarf bean, potato tuber, and leaves of tobacco, dwarf bean, horseradish, sugar beet and broccoli had lower activities.

Knox & Mehler (1950) found that the oxidation of tryptophan in liver extracts of several animals was accelerated by addition of enzymes and substrates producing hydrogen peroxide. This has now been found to be the case with pea-seedling tissues and extracts. Oxidation depended on the supply of enzymically generated peroxide which was used in a 'coupled oxidation'. The oxidation of aliphatic amines by amine oxidase (Kenten & Mann, 1952) provided a convenient source of peroxide.

$$R.CH_2NH_2 + O_2 + H_2O \xrightarrow{\text{amine oxidase}} R.CHO + NH_3 + H_2O_2. \quad (1)$$

The peroxide formed in reaction (1) was usually decomposed by catalase so that 0·5 mole oxygen was used per mole amine oxidized. When tryptophan was added, even in the presence of catalase, it combined with peroxide from reaction (1) according to reaction (2) thus increasing the oxygen consumption

$$\text{Tryptophan} + 2H_2O_2 \xrightarrow{\text{peroxidase}} \text{Products}. \quad (2)$$

The oxidation of tryptophan by plant peroxidase, which has been reported briefly by Sizer (1947), is the subject of this paper.

METHODS AND MATERIALS

Plant material. Pea seedlings were grown for 7–10 days either (*a*) in dishes of moist sand kept in the dark or (*b*) in seedboxes of John Innes potting compost in a greenhouse with normal lighting. Method (*a*) gave better growth in winter. The seedlings were washed at harvest to remove sand or soil and any diseased parts were discarded. Where indicated in the Experimental section they were dissected with scissors into root, cotyledon and epicotyl portions.

Tissue slices. Tissues were cut freehand with a razor blade moistened with 0·067 M-potassium phosphate buffer at pH 7. Growing tips of roots and epicotyls were discarded. Slices from a number of plants were pooled and washed thrice with buffer, then suspended in the same buffer saturated with toluene.

Tissue extracts. Fresh tissue was weighed and minced and the juice pressed through cloth. The residue was extracted by grinding for 2 min. in a Townson and Mercer (Croydon) macerator with 0·067 M buffer equal to the fresh weight; the extract was pressed through cloth and combined with the juice. Large particles were removed by centrifugation at 1000 rev./min. for 5 min.

Enzymes. Catalase was prepared from human erythrocytes by Bonnichsen's method (1947). Purified liver catalase, notatin and horseradish peroxidase were presented by Dr E. F. Hartree. Peroxidase activity was measured by Keilin & Hartree's method (1951) except that an EEL colorimeter (Evans Electroselenium Ltd.) and filter 622 were used instead of a Spekker absorptiometer. The number of enzyme units (E.U.) in a preparation $= Vw/1000v$, where V is the total volume of solution, w = mg. purpurogallin formed by v ml. of solution.

Dry weight. Tissue was dried to constant weight at 110°.

Deproteinization. Samples were precipitated either (*a*) by addition of trichloroacetic acid solution to a final concentration of 5% (w/v) or (*b*) by addition of an equal volume of 5% $(CH_3COO)_2Zn$, $2H_2O$ and half the volume of 0·4 N-NaOH, and filtered or centrifuged.

Analytical methods. Tryptophan was estimated colorimetrically (Horn & Jones, 1945) by reaction with *p*-dimethylaminobenzaldehyde (Ehrlich reagent) in acid solution. This reaction is not specific for tryptophan. Tryptamine gives an equivalent amount of the same blue colour, and colours are given by all pyrroles with at least one free methene hydrogen, by indoles and by furans (Reichstein, 1932). Since it was found that the blue colour was readily bleached by H_2O_2, catalase was added to decompose peroxide before addition of Ehrlich reagent. In some experiments duplicate estimations of tryptophan were made by Eckert's method (1943).

Ammonia was estimated (*a*) by steam-distillation in the Markham apparatus, or (*b*) by diffusion in Conway dishes at room temperature. Method (*b*) was used when putrescine was included in the reaction mixture because the product of its oxidation is a steam-volatile base (Kenten & Mann, 1952).

Total nitrogen was estimated by the Kjeldahl method.

Manometric measurements. O_2 uptake and CO_2 production were measured in the Warburg apparatus in air at 28°. The total volume of each reaction mixture was 3 ml., and KOH was present in the centre wells, except when CO_2 was measured (by addition of 0·2 ml. of 2 N-HCl at the end of the incubation).

RESULTS

Tryptophan oxidation by tissue slices

The respiration of pea-seedling slices was slow (Q_{O_2} 1 to 4, Table 1). It was increased by addition of substrates for amine oxidase, such as putrescine or ethanolamine, but not by tryptophan. In the presence of amines, addition of tryptophan increased the oxygen consumption, and part of the tryptophan was oxidized. The rate of oxidation averaged 0·5 μmole/g. dry wt./hr. without amine, and 0·8 μmole/hr. with amine, when the tryptophan concentration was 0·00025 M. At 0·004 M-tryptophan, the averages were 4·6 and 9·1 μmoles/hr. Tryptophan oxidation was followed in deproteinized filtrates by colorimetry and by the decrease in absorption at 279 mμ. No product was detected, and no colour was formed.

Tryptophan oxidation by tissue extracts

Tryptophan was oxidized at about the same rate by whole tissue extracts as by slices (Table 2) and the oxidation was more definitely accelerated by ethanolamine. This amine rather than putrescine was used for most of the experiments with extracts

because the product of oxidation of putrescine reacts with Ehrlich reagent to give a red compound which interfered with the tryptophan assays. Ethanolamine was oxidized comparatively slowly so that a long incubation was necessary. Toluene was added to control bacterial infection.

Table 1. *Oxidation of tryptophan by pea-seedling tissue slices*

(Slices shaken at room temperature in 25 ml. flasks containing 5 ml. 0·067 M-phosphate buffer, pH 7, saturated with toluene. Expt. 1: 0·5 g. slices, 1·25 μmoles tryptophan, 50 μmoles amine, incubated 16 hr. Expt. 2: 1·0 g. slices, 20 μmoles tryptophan, 1 m-mole amine, incubated 12 hr. Q_{O_2} = μl. O_2/mg. dry wt./hr.)

Tissue	Q_{O_2}	Additions	Tryptophan oxidized (μmoles) Expt. 1	Expt. 2
Root	4·0	None	0·22	4·44
		Putrescine	0·37	.
		Ethanolamine	0·54	6·52
Cotyledon	1·0	None	0·22	2·35
		Putrescine	0·29	.
		Ethanolamine	0·35	7·00
Epicotyl	2·6	None	0·25	3·09
		Putrescine	0·30	.
		Ethanolamine	0·54	5·80

Table 2. *Oxidation of tryptophan by pea-seedling extracts*

(Extract equivalent to 15 g. fresh wt. of tissue shaken at room temperature in 100 ml. flasks containing 20 μmoles tryptophan, 1·5 m-moles ethanolamine, 0·067 M-phosphate buffer, pH 7, and water to 30 ml.)

Tissue	Additions	Tryptophan oxidized (μmoles) 6 hr.	16 hr.
Root	None	0·2	3·6
	Ethanolamine	5·3	10·2
Cotyledon	None	0·6	3·3
	Ethanolamine	8·5	16·2
Epicotyl	None	0	3·3
	Ethanolamine	7·5	15·4

The rate of oxidation by extracts was decreased by ageing or by dialysis, and was restored by addition of ethanolamine. The extract remained active for 1 week if saturated with toluene and stored at +2°.

Absorption spectra of deproteinized and diluted reaction mixtures of cotyledon extract are plotted in Fig. 1. The usual spectrum of tryptophan persisted in the control without ethanolamine. The product in the mixture containing ethanolamine, in which most of the tryptophan had been oxidized, showed new absorption maxima at 270 and 370 mμ.

Purification of the tryptophan-oxidizing enzyme system

Centrifugation. Nearly all the activity in extracts was associated with soluble enzymes. In a typical experiment, extract was centrifuged for 30 min. at

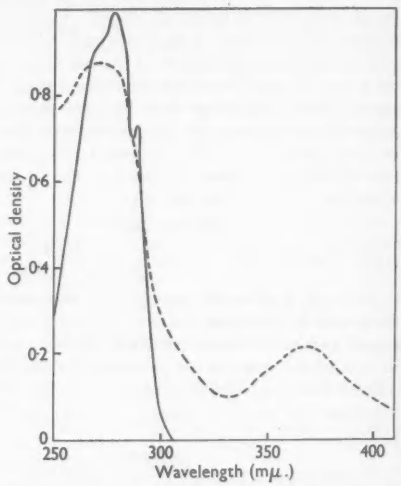

Fig. 1. Absorption spectra of tryptophan (continuous line) and of its oxidation product (interrupted line) in whole pea-seedling extract.

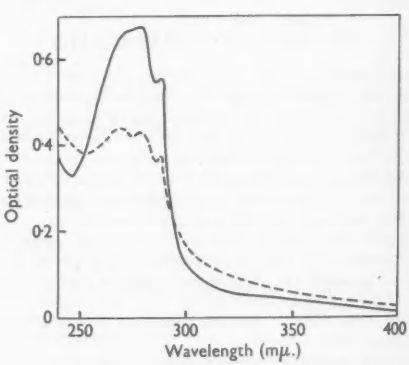

Fig. 2. Absorption spectra of tryptophan before (continuous line) and after (interrupted line) oxidation by enzyme *R*3.

10 000 *g*. Tryptophan-oxidizing activity and peroxidase were assayed in samples of the clear supernatant (*S*1) and of the residue (*R*1) resuspended in one-tenth of the original volume of 0·067 M-phosphate buffer. *S*1 contained 90 % of the peroxidase and of the tryptophan-oxidizing activity.

There was no increase of activity of *S*1 when *R*1 was added back to it. Subsequent steps in purification started with *S*1.

Precipitation with ammonium sulphate. *S*1 was fractionated by addition of ammonium sulphate to one-third and two-thirds saturation. The two precipitates and the supernatant at two-thirds saturation were examined for activity and protein nitrogen after dialysis to remove ammonium sulphate. The recoveries, based on activity and protein nitrogen of *S*1 were: 17% of activity and 36% of protein precipitated at one-third saturation; 34% of activity and 32% of protein precipitated between one-third and two-thirds saturation; 37% of activity and 13% of the protein in the supernatant at two-thirds saturation.

Precipitation with immiscible solvents. A great deal of inactive protein could be removed before ammonium sulphate precipitation by saturation of *S*1 with *n*-butanol (Morton, 1950) or better by addition of chloroform and ethanol. 0·25 Vol. of *n*-butanol, or 0·1 vol. of chloroform and 0·2 vol. of ethanol, were added to *S*1 with stirring. The organic layer and the bulky protein precipitate which floated at the interface on centrifugation were discarded. About half of the activity was recovered in the aqueous layer (*S*2) after butanol or chloroform-ethanol treatment, but the latter method was more often used because it removes more inactive protein.

About 40 g./100 ml. of ammonium sulphate was required to saturate *S*2 as compared with 70 g./100 ml. for *S*1. The precipitate (*R*2) so obtained contained most of the amine oxidase, but did not oxidize tryptophan. Peroxidase was not precipitated in *R*2 (Table 3). If the organic solvent was first removed from *S*2 by dialysis, more ammonium sulphate could be dissolved, and the precipitate between one-third and two-thirds saturation contained amine oxidase, together with peroxidase and most of the tryptophan-oxidizing activity. This enzyme (*R*3), after dialysis, was a yellow solution which darkened on standing. It was dried over sodium hydroxide by evaporation in a desiccator under reduced pressure. An attempt to prepare a dry powder by addition of 2 vol. of acetone resulted in loss of activity on ethanolamine and on tryptophan.

Separation of components of the enzyme system

Several attempts were made to separate pea-seedling peroxidase from amine oxidase. A solution of *R*3 was carefully adjusted to pH 5·5. The precipitate (*R*4) was dissolved in phosphate buffer at pH 7 and the supernatant (*S*3) was readjusted to pH 7. The peroxidase and tryptophan-oxidizing activities of *S*1, *S*2, *S*3, *R*2, *R*3 and *R*4 are compared in Table 3. Most of the amine oxidase was precipitated at pH 5·5, while the peroxidase and tryptophan-oxidizing activity were not. Rather more efficient separation was achieved by treatment of *R*3 with calcium phosphate gel (prepared according to Singer & Kearney, 1950) at pH 5·5. Peroxidase (*P*) was not adsorbed, while amine oxidase (*A*) could be recovered by elution from the gel with phosphate buffer pH 8·5. Neither enzyme alone

Table 3. *Tryptophan oxidation and peroxidase activity in pea-seedling extracts purified in several ways*

(Enzymes prepared as described in the text, shaken at room temperature in flasks containing 20 μmoles tryptophan, 1·5 m-moles ethanolamine and 0·067 M-phosphate buffer at pH 7. Activity expressed as μmoles tryptophan oxidized/ml./hr. and peroxidase as E.U./100 ml.)

	Tryptophan-oxidizing activity		Peroxidase	
Enzyme	Expt. 1	Expt. 2	Expt. 1	Expt. 2
*S*1	0·9	0·7	4·56	5·3
*S*2	0·4	.	1·99	.
*S*3	.	0·8	.	6·1
*R*2	0·04	.	0·07	.
*R*3	2·2	.	4·3	.
*R*4	.	0·1	.	0·8

Table 4. *Tryptophan oxidation by amine oxidase and peroxidase from pea seedlings*

(Enzymes *A* (amine oxidase) and *P* (peroxidase) prepared as described in the text. Warburg vessels contained enzyme as indicated, 10 μg. catalase, 20 μmoles ethanolamine, 0·067 M-phosphate buffer, pH 7.)

Enzyme (ml.)			Tryptophan (μmoles)	Oxygen uptake (μmoles)		
A	*P*	Boiled *P*		Expt. 1	Expt. 2	Expt. 3
0·2	.	.	.	2·6	4·6	.
0·2	.	.	25	2·8	5·6	.
.	1·5	.	.	0·4	0·8	.
.	1·5	.	25	0·5	1·2	.
0·2	1·5	.	.	2·9	5·3	.
0·2	1·5	.	25	4·7	8·3	.
.	.	1·5	.	.	.	0·3
.	.	1·5	25	.	.	0·4
0·2	.	1·5	.	.	.	3·0
0·2	.	1·5	25	.	.	3·1

oxidized tryptophan. Oxidation by the reconstituted enzyme was followed by the oxygen uptake in Warburg manometers (Table 4). Boiled peroxidase preparation was inactive.

Course of the reaction

It was found impossible to measure accurately the gas exchange during tryptophan oxidation by *S*1 because of the rapid respiration of the enzyme itself. Dried enzyme *R*3 retained reasonable activity with substrates added, while its blank respiration was about 1 % of that of *S*1. Enzyme *R*3 diluted to contain about 1 mg. protein nitrogen/ml. was used in the following experiments.

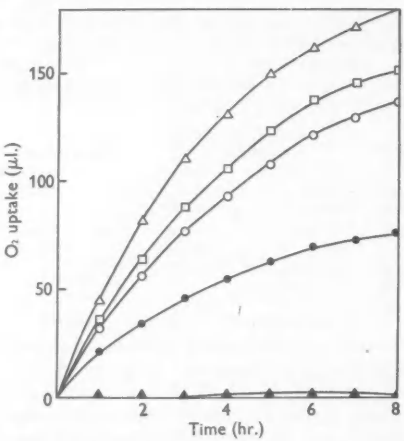

Fig. 3. Coupled oxidation of tryptophan during oxidation of ethanolamine by enzyme *R*3. Warburg vessels contained 1 ml. *R*3, 10 μg. catalase, 10 μmoles ethanolamine and 0·067 M-phosphate buffer, pH 7. The oxygen uptake of the enzyme has been subtracted. No tryptophan, ●—●; 5 μmoles tryptophan, ○—○; 10 μmoles tryptophan, □—□; 37·5 μmoles tryptophan, △—△; 37·5 μmoles tryptophan without ethanolamine, ▲—▲.

Requirement for hydrogen peroxide. It is apparent from the experiment presented in Fig. 3 that tryptophan oxidation is dependent on a supply of hydrogen peroxide. The oxygen consumption of enzyme with tryptophan was almost the same as that of enzyme alone. Oxygen uptake with ethanolamine was doubled in the presence of sufficient tryptophan. This implies that peroxidase competed successfully with catalase for the peroxide. The amount of peroxide so utilized depended on the concentrations of tryptophan (Fig. 3) and of enzyme (Fig. 4). The reaction mixture in both experiments contained 10 μg. added catalase besides that already present in the enzyme, which was by itself enough to destroy all the peroxide formed. Higher catalase concentrations had no effect on oxygen uptake with ethanolamine, but inhibited the extra oxygen uptake with tryptophan, supporting the inference that peroxide is essential. Oxidation was inhibited by 31 % by 50 μg. catalase, 52 % by 100 μg. and 76 % by 500 μg. catalase.

Oxygen consumption. Results from several experiments using different preparations of enzyme (Table 5) indicate that 1 mole of tryptophan is oxidized with the uptake of 1 mole of oxygen. The oxygen uptake with tryptophan was never more than double that with ethanolamine alone, even when the tryptophan concentration was high compared with that of ethanolamine. The reaction is therefore between 1 molecule of tryptophan and

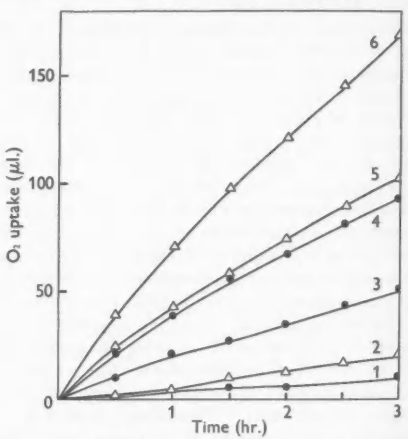

Fig. 4. Coupled oxidation of tryptophan. Warburg vessels contained 10 μg. catalase, 10 μmoles ethanolamine and 0·067 M-phosphate buffer, pH 7. Curve 1, 0·1 ml. *R*3; curve 2, 0·1 ml. *R*3 + 25 μmoles tryptophan; curve 3, 0·5 ml. *R*3; curve 4, 0·5 ml. *R*3 + 25 μmoles tryptophan; curve 5, 1·0 ml. *R*3; curve 6, 1·0 ml. *R*3 + 25 μmoles tryptophan.

2 molecules of hydrogen peroxide (reaction 2 above).

Production of ammonia and carbon dioxide. The amount of ammonia formed when tryptophan was oxidized with ethanolamine as the peroxide donor was the same as that due to oxidation of ethanolamine without tryptophan (Table 5). When the peroxide came from notatin and glucose (Table 6) no ammonia was formed. Less than 0·25 mole of carbon dioxide was formed per mole of tryptophan oxidized.

The oxidation product. The product of tryptophan oxidation by purified extracts is not that formed in whole extract. The most striking difference is that in the purified extract the product is coloured reddish brown, a feature which was first observed by

Elliott (1932*a*) for milk peroxidase. Elliott (1932*b*) was unable to oxidize tryptophan with horseradish peroxidase, and considered this an important difference between the two enzymes. The absorption spectrum of the product (Fig. 2) did not show maxima at 270 and 370 mμ. The changes in absorption were similar to those observed by Weil, Gordon & Buchert (1951) in the photochemical oxidation of tryptophan catalysed by methylene blue. The photochemical oxidation also involved hydrogen peroxide, which was formed by reversible oxido-reduction of the dye. The extent of decomposition of the indole nucleus was more extensive in that 4 moles of oxygen were consumed and 2 moles of carbon dioxide and 0·3 mole of ammonia produced per mole of tryptophan oxidized. The site of attack was considered to be the five-membered ring of the indole nucleus, because the absorption maximum at about 280 mμ., due to the ethylenic linkage in the pyrrole ring conjugated with the benzene nucleus, disappeared, while a new maximum appeared at 260 mμ. suggesting that the benzene chromophore persisted in the oxidation product. The indole nucleus of tryptophan is also destroyed by irradiation with cathode rays (Proctor & Bhatia, 1953), but in this case considerable amounts of ammonia are released.

Effect of pH

The variation of activity of enzyme *R*3 with pH was tested in 0·067 M potassium phosphate buffers from pH 6 to 8·5. Oxygen uptake was fastest at pH 7 and the rate fell off more sharply on the acid than on the alkaline side.

Effect of inhibitors

Tryptophan oxidation was completely inhibited by 10^{-3}M-potassium cyanide at pH 7. It was not inhibited by 10^{-6}M-sodium azide, but was inhibited to the extent of 8% at 10^{-4}M, 73% at 10^{-3}M and 95% at 10^{-2}M. Ethylenediaminetetraacetic acid (Sequestrene NA2, Alrose Chemical Co.,

Table 5. *Gas exchange in tryptophan oxidation by pea-seedling enzymes*

(Warburg vessels contained 1 ml. enzyme *R*3, 20 μg. catalase, 0·067M-phosphate buffer, pH 7. Deductions have been made for oxygen uptake and ammonia and carbon dioxide formed with ethanolamine alone, and for oxygen uptake, ammonia and carbon dioxide formed and tryptophan oxidized with tryptophan alone.)

Additions		Oxygen uptake (μmoles)	Ammonia formed (μmoles)	Carbon dioxide formed (μmoles)	Tryptophan oxidized (μmoles)
Ethanolamine (μmoles)	Tryptophan (μmoles)				
5	5	1·1	0	.	0·9
5	20	2·3	0	.	1·8
10	5	1·6	0	.	1·2
10	10	1·8	0	.	2·3
10	10	2·8	.	0·7	.
10	10	3·6	.	0·4	.
10	10	2·3	.	0·5	.
10	15	2·6	0	.	2·3
10	15	4·9	0	.	4·0
10	20	2·5	0	.	2·8
10	20	2·3	.	0·4	.
10	20	3·0	.	0·7	.
20	10	6·5	.	.	6·7

Table 6. *Tryptophan oxidation by purified peroxidases with notatin and glucose*

(Warburg vessels contained 20 μg. catalase and 0·067M-buffer, pH 7, 20 μg. notatin, 22 μg. horseradish peroxidase, 1 ml. pea-seedling peroxidase, 20 μmoles glucose and 10 μmoles tryptophan where indicated.)

Additions					Oxygen uptake (μmoles)	Tryptophan oxidized (μmoles)
Notatin	Horseradish peroxidase	Pea-seedling peroxidase	Glucose	Tryptophan		
−	+	−	+	−	1·6	.
−	+	−	+	+	2·4	2·5
+	−	−	+	−	10·9	.
+	−	−	+	+	11·5	1·2
+	+	−	+	−	12·0	.
+	+	−	+	+	18·0	4·2
+	−	+	−	−	0	.
+	−	+	−	+	1·1	1·6
+	−	−	+	−	8·4	.
+	−	−	+	+	9·1	0·2
+	−	+	+	−	11·2	.
+	−	+	+	+	15·6	3·6

Providence, R.I.) inhibited 21% at 10^{-3}M, 44% at 2×10^{-3}M and 100% at 5×10^{-3}M. The inhibition by potassium cyanide was in part attributable to inhibition of amine oxidase. Sodium azide and Sequestrene had very little effect on this enzyme; they inhibited peroxidase.

Specificity of reaction

Both optical isomers of tryptophan were oxidized at the same rate and neither was oxidized by *R*3 without peroxide. Although indolylacetic acid was oxidized rapidly, its oxidation did not depend on peroxide; the oxygen uptake with ethanolamine and indolylacetic acid was the sum of that with the two substrates separately. Only the side chain was oxidized, as found by Tang & Bonner (1947), since the product retained the absorption spectrum typical of indoles and gave a blue colour with Ehrlich reagent, but did not give the red colour characteristic of indolylacetic acid with Salkowski reagent.

Tryptophan oxidation in model systems containing purified peroxidases

Hydrogen peroxide decomposed slowly with either or both tryptophan and horseradish peroxidase (Table 7). The decomposition stopped after 2 hr. and the Warburg vessels were incubated for a further 4 hr. without change in manometer reading. Catalase (10 μg.) was then added from the side arms and the oxygen output measured. The total oxygen output before and after addition of catalase was in all cases nearly the same; it is concluded that there was no significant reaction between tryptophan and peroxide. All the reaction mixtures were colourless at the end of this experiment, but solutions of tryptophan in stronger (0·05M) peroxide became yellow after 12 or more hr. at room temperature, and in such solutions the absorption at 279 mμ. decreased. The reaction was not apparently accelerated by peroxidase. These observations are consistent with Elliott's conclusion that horseradish peroxidase does not oxidize tryptophan. However, when peroxide was added slowly to a solution of tryptophan and horseradish peroxidase, so that the peroxide concentration was low, tryptophan oxidation could be measured. In one experiment a mixture of 704 μg. horseradish peroxidase, 70 μg. catalase, 5 μmoles tryptophan, 0·067M-phosphate buffer at pH 7 and water to 20 ml. was stirred in a water bath at 28°, while 0·01M-hydrogen peroxide was run in from a horizontal burette with capillary delivery. The rate of addition of peroxide was 0·2 μmole/min. Disappearance of tryptophan was followed in successive samples from which peroxide was removed by addition of 7 μg. catalase. 27% of the tryptophan was oxidized in 6·5 hr. The red-brown colour of the product was noticed after 2 hr.

Table 7. *Reaction of tryptophan and peroxidase with hydrogen peroxide*

(All Warburg vessels contained 20 μmoles hydrogen peroxide and 0·067M-phosphate buffer, pH 7, 100 μg. horseradish peroxidase and 10 μmoles tryptophan where indicated. 10 μg. catalase added to all vessels after 6 hr.)

Additions		Oxygen output (μmoles)		
Peroxidase	Tryptophan	Before adding catalase	After adding catalase	Total
−	−	0·1	18·5	18·6
+	−	1·7	16·3	18·0
−	+	2·5	15·4	17·9
+	+	4·4	12·9	17·3

Tryptophan oxidation was also demonstrated in a coupled enzyme system in which peroxide was provided from the oxidation of glucose by glucose oxidase (notatin) and used by purified horseradish peroxidase (Table 6). The relation of oxygen used to tryptophan oxidized and the alteration in the absorption spectrum were the same as with the pea-seedling enzymes. Both D- and L-tryptophan were oxidized at the same rate, while indolylacetic acid, glutamic acid, alanine, tyrosine and histidine were not oxidized. Tryptophan was oxidized by pea-seedling peroxidase preparation *P* using notatin and glucose as peroxide source (Table 6).

Presence of peroxidase in pea seedlings

Pea seedlings contained about the same concentration of peroxidase as horseradish. The enzyme was present in all parts, but especially in the roots and cotyledons (Table 8). From the data of Table 6, 1 E.U. of purified horseradish peroxidase can oxidize

Table 8. *Distribution of peroxidase in 10-day-old etiolated pea seedlings*

Tissue	Fresh wt. (g.)	Dry wt. as % fresh wt.	Peroxidase content E.U.*	E.U./g. fresh wt.	E.U./g. dry wt.
Root	100	5·8	2·94	0·0294	0·507
Cotyledon	124	28·2	7·24	0·0584	0·207
Epicotyl	164	5·9	1·72	0·0105	0·177

* E.U. = enzyme units.

52·2 μmoles of tryptophan/hr. The average content of peroxidase in pea-seedling tissue was 0·03 E.U./g. fresh wt., so that 1 g. fresh tissue might be expected to oxidize 1·6 μmoles tryptophan/hr. The rates observed (Table 1) were about one-third as fast.

DISCUSSION

It has been shown that tryptophan oxidation by tissues and extracts of pea seedlings is increased by addition of certain amines. This acceleration is brought about by hydrogen peroxide formed during oxidation of the amines by the amine oxidase, for amine and amine oxidase can be replaced by glucose and notatin or by a suitable concentration of hydrogen peroxide. Since the activity of extracts purified in several ways is parallel to their peroxidase content, and the same reaction is catalysed by purified horseradish or pea-seedling peroxidase, it is clear that tryptophan is oxidized by peroxidase in a coupled enzyme system using enzymically generated peroxide. The peroxidase reaction occurs in the presence of the normal concentration of catalase but is inhibited by higher concentrations of catalase.

Oxidation in tissues and whole extracts produced no coloured substance; a product with absorption maxima at 270 and 370 mμ. accumulated in whole extracts. It differed from tryptophan in that the red compound it formed with Ehrlich reagent did not change to blue on addition of sodium nitrite. This property is shared by 3-hydroxykynurenine (Makino, Satoh, Fujiki & Kawaguchi, 1952), which has absorption maxima at 268·5 and 370 mμ., and is a product of tryptophan oxidation in the rat (Dalgliesh, 1952).

In purified extracts and in model systems with horseradish peroxidase, a coloured substance possessing no definite absorption spectrum was formed. No ammonia and very little carbon dioxide was produced. The amount of tryptophan oxidized depended on the amount of peroxide supplied, and the oxygen uptake was equivalent to 2 moles hydrogen peroxide (1 mole oxygen)/mole of tryptophan oxidized.

In some respects these results resemble those obtained by Knox & Mehler (1950) for tryptophan oxidation in liver extracts. Both oxidations depend on a peroxide-donating system. The amount of peroxide required is comparable with the tryptophan oxidized and its concentration must be low. Both are inhibited by catalase and in both the oxygen uptake is 1 mole/mole of tryptophan oxidized. Both are catalysed by enzymes which are not sedimented at 8000 rev./min. On the other hand, the pea-seedling enzyme is not specific to the L-isomer, and the animal enzyme does not oxidize typical peroxidase substrates, though it is inhibited by peroxidase inhibitors. The oxygen uptake of the liver tryptophan peroxidase, using D-alanine as source of hydrogen peroxide, was sometimes more than doubled by addition of tryptophan. This result led to the interpretation of the reaction as taking place in two steps, the second step providing peroxide which was used in the first, thus permitting 'cyclic oxidation'. Tryptophan never more than doubled the oxygen uptake with pea-seedling enzyme, even with several times more tryptophan than peroxide donor. The amount of tryptophan oxidized was strictly dependent on the peroxide available. There was no 'sparking' effect by peroxide, and a second reaction producing peroxide is improbable.

In spite of these differences, it is possible there may be some relation between the mechanism in the plant and animal. It may be suggested that in the plant tryptophan is oxidized by a series of enzymes of which peroxidase is the first, using two molecules of hydrogen peroxide. The product of this first reaction may be converted to 3-hydroxykynurenine by enzymes which are less stable or less active than peroxidase and which had been removed from the purified extracts used in this work. There is sufficient peroxidase in pea-seedling tissue to account for all the tryptophan oxidation observed. The rates averaged about 0·5 μmole/g. fresh wt./hr., which is comparable with the rate in normal rat liver (Knox, 1951) and more than 100 times as fast as the conversion of tryptophan to indolylaceticacid in tobacco ovaries (Wildman & Muir, 1949). Oxidation by peroxidase could therefore be the normal fate of tryptophan in this plant tissue.

SUMMARY

1. The oxidation of tryptophan in tissue slices and extracts from pea seedlings was accelerated by addition of certain amines.

2. The tryptophan-oxidizing activity of extracts was associated with peroxidase.

3. In the whole extract, the product of oxidation was a substance with absorption maxima at 270 and 370 mμ.

4. In purified extract two molecules of hydrogen peroxide oxidized one molecule of tryptophan to a coloured product.

5. Tryptophan was oxidized in model systems by purified horseradish peroxidase with free hydrogen peroxide or with peroxide-forming enzymes and substrates.

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The Colorimetric Estimation of Formaldehyde by Means of the Hantzsch Reaction

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Most of the colour reactions described for formaldehyde require severe conditions incompatible with the preservation of biological material, although consistent results can be obtained where complete destruction of this material can be tolerated (MacFadyen, 1945). Examples of such reactions are those employing chromotropic acid in hot strong sulphuric acid (Eegriwe, 1937), phenylhydrazine and ferricyanide in strong hydrochloric acid (Kersey, Maddocks & Johnson, 1940) and various tests quoted by Walker (1944), some using alkaloids and strong mineral acid and others using phenols and strong alkali. It would be convenient to have at least one colorimetric reaction that could be carried out under less violent conditions. It might also be desirable to preserve biological material on which an estimate of formaldehyde content was required, and from which the taking of samples was inconvenient or impossible.

In the course of work on the neutralization of formaldehyde in living bacterial suspensions, it was found that a yellow colour developed with acetylacetone in the presence of ammonium salts and, less rapidly, in the presence of glycine. This appeared to give a possible alternative to the reactions mentioned above and, as has already been briefly reported (Nash, 1952), was traced to the formation of 3:5-diacetyl-1:4-dihydrolutidine. Two-stage syntheses of this substance have been described using concentrated reagents and catalysts (Scholtz, 1897; Schneider & Sanger, 1903), but for the particularly simple molecules concerned the reaction can be quantitative for traces of formaldehyde under quite mild conditions.

General description of the reaction and its product

When traces of formaldehyde are added to approximately neutral solutions of acetylacetone and ammonium salt, a yellow colour gradually develops owing to the synthesis of diacetyldihydrolutidine (DDL). Under optimum conditions the molecular extinction in terms of formaldehyde has a smooth maximum of 8000 at 412 mμ., independent of dilution. The absorption curve is shown in Fig. 1, and was obtained using reagent *A* (see below); this has since been somewhat modified, but the effect on the absorption curve should be negligible. It can be seen that a good separation is obtained between the absorption maximum of DDL and the first absorption band of acetylacetone. The position of this curve coincided with that obtained from solutions of recrystallized DDL itself in

water, but the molecular extinction of the latter was never greater than 7700. This difference is probably due to changes in the crystals, since after keeping they become more and more difficult to dissolve.

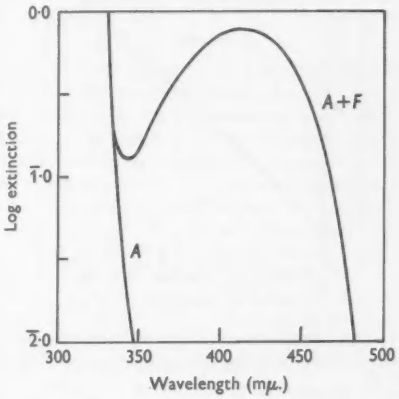
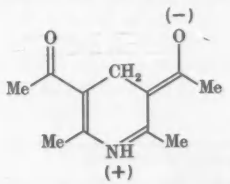


Fig. 1. Extinction due to 10^{-4} M-formaldehyde left standing for 5 hr. at 20° with M-ammonium acetate/0·05 M-acetylacetone/0·03 M-acetic acid ($A + F$) and the cut-off at the shorter wavelengths due to the control solution (A).

The DDL molecule is probably polar, both in solution and in the crystals. The structure (*a*) accounts satisfactorily for its insolubility in ether,



its high melting point (about 200°) and the negligible effect of pH changes on the extinction, over the range 4–10. The two original acetylacetone molecules can be seen to be linked by the formaldehyde methylene at the top and the ammonia nitrogen at the bottom. One of the possible mesomeric structures is shown; the net charge separation is probably a good deal less than unity.

Effect of pH *and reagent composition*

The reaction at low formaldehyde concentrations appears to be most nearly quantitative at pH 5·5–6·5. In one experiment with ammonium phosphate buffer kept molar in ammonia, and with 0·01 M-acetylacetone, the relative yields measured on a colorimeter were: pH 4, 75 %; pH 5, 94 %; pH 6, 100 %; pH 7, 96 % and pH 8, 87 %. The effect of varying the reagent composition is shown in Table 1, which gives the efficiency as a percentage of that obtained with reagent *B* (see below). The approximate times for 99 % of the actual reaction at any composition are also given, deduced from absorptiometer readings by assuming a first-order law for the final stages of the reaction, i.e. that the rate of colour development is proportional to the amount remaining to be developed. Owing to fading, estimates for the longer times are not possible, but such solutions would not be generally used. It can be seen that the efficiency falls off much more rapidly with decreasing ammonia concentration than with decreasing acetylacetone concentration. In fact, high yields of DDL can be obtained with only traces of acetylacetone and of formaldehyde, provided that sufficient ammonium salt is present. The practical range, however, can be seen to be restricted to ammonia concentrations between M and 0·1 M and acetylacetone concentrations between 0·1 and 0·001 M.

Preparation and use of reagent

It was found that colour production was slightly greater from acetate solutions than from phosphate. Since acetate solutions are usable in the presence of calcium and magnesium salts, if necessary, the following mixture should be of general use

Table 1. *Concentrations of diacetyldihydrolutidine reached in 24 hr. at 20° in solutions of different compositions, but with the same initial amount* (10^{-4} M) *of formaldehyde*

(Slight excess of acetic acid was present, to the extent of 0·05 mol. prop. of the ammonium acetate throughout. Figures are percentages of the value obtained with the standard solution containing M-ammonia and 0·01 M-acetylacetone. Where possible the estimated times, in hours, to attain 99 % of the maximum colour developed, are given in parentheses.)

Strength of acetylacetone	Strength of ammonium acetate				
	3 M	M	0·33 M	0·1 M	0·033 M
0·1 M	99 (3)	97 (4)	94 (16)	81	44
0·033 M	103 (3)	100 (4)	97 (14)	88	48
0·01 M	103 (7)	100 (5)	100 (13)	90	51
0·0033 M	102 (11)	101 (8)	98 (15)	89	53
0·001 M	98 (16)	98 (16)	95	79	45
0·00033 M	74	88	77	61	35

(analytical grade reagents, redistilled acetylacetone, which should be colourless):

Reagent B

2 M-ammonium acetate or 150 g. approx.
0·05 M-acetic acid or 3 ml.
0·02 M-acetylacetone or 2 ml.
} to 1 l. water.

An earlier mixture, referred to as reagent *A*, contained five times as much acetylacetone, but this was found to be unnecessarily strong. Some of the results described below were obtained with reagent *A*, but the difference is negligible; the main reasons for reducing the acetylacetone concentration were that the zero absorption would be less (see below on sensitivity), that the reagent should keep longer (see below on stability) and that the reagent should be less toxic to living material.

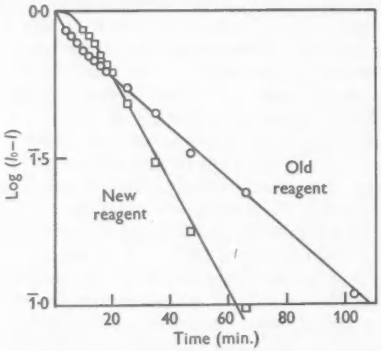


Fig. 2. The development of colour with time using freshly made ammonium acetate-acetylacetone reagent, and 1-day-old reagent, on addition of 10^{-4} M-formaldehyde. Colour development plotted as a first-order reaction; I_0 is the maximum colour developed, I is the colour at a given time.

The reagent is mixed with an equal volume of solution containing not more than 8 μg. of formaldehyde per ml. (if there is more, DDL will eventually precipitate in long, yellow, flexible crystals) and the colour estimated on a photoelectric colorimeter with a deep-blue filter. From Table 1 the time for 99 % reaction is 5 hr. at 20°. To a first approximation the reaction can be treated as unimolecular (Fig. 2), and in fact the times for 99 % completion, calculated from unimolecular plots, follow the Arrhenius relation closely (Fig. 3). The reduction in the time required for 99 % completion at higher temperatures can then be estimated; for example, reagent *B* at 37° should take only 40 min. and at 58°, 5 min. The shape of the early part of the reaction curve is also changed as the temperature is raised (Fig. 4).

If the reaction is considered for the detection, rather than the estimation, of formaldehyde, it should be noted that all the components of the reagent are volatile, and that DDL crystals are strongly fluorescent under soft ultraviolet light.

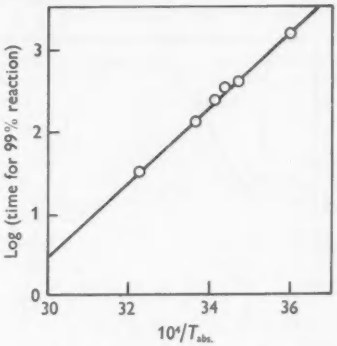


Fig. 3. The temperature coefficient of the reaction between acetylacetone, ammonia and formaldehyde between 5° and 37°.

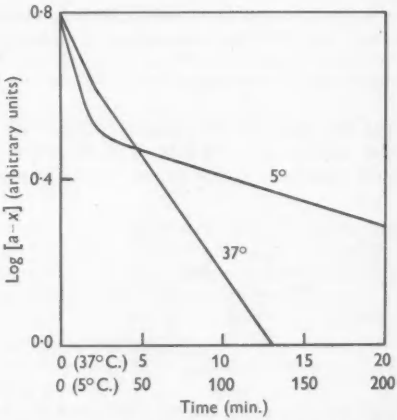


Fig. 4. Rate of production of colour from acetylacetone (0·05 M), ammonium acetate (M) and formaldehyde (10^{-4} M) at two different temperatures. Initial stages, using 1-day-old reagent, plotted as a first-order reaction; a is the maximum final colour developed, x the amount at any given time.

Stability of reagent

The addition of strong ammonium acetate to solutions of acetylacetone causes a slow shift of the edge of the first ultraviolet absorption band towards the visible. This shift has stabilized in 24 hr. at about 18 mμ. from its original position, the solution still remaining colourless to the eye. The extent to which the shift has proceeded at intermediate times has no effect on the final absorption in the blue caused by the addition of formaldehyde,

but merely affects the shape of the reaction curve (Fig. 2). It was found that after 2 weeks at 20°, reagent *A* developed a feral odour and readings from it had dropped to 92% of those from fresh reagent. Reagent *B*, on the other hand, appeared to be unchanged.

Sensitivity

For a given amount of formaldehyde the extinction in the present method is only one-third of that obtained in the chromotropic acid reaction at 570 mμ. (MacFadyen, 1945). However, this is not a true measure of comparative sensitivity because the absorption of control solutions is higher for chromotropic acid. MacFadyen (1945) states that it is between 9 and 25% for analytical grade reagent. This was confirmed in this laboratory, and even after a long and tedious purification it was still 4%/cm. On the other hand, reagent *B* has a control absorption of only 1%, which does not increase appreciably for a week or so if the reagent is protected from traces of formaldehyde. When long light paths are possible, reagent *B* is probably about as sensitive as chromotropic acid.

Measurement of colour

The calibration curve from absorptiometer readings is not convertible to a straight line by plotting log. intensity of transmitted light against concentration. This is not due to any detectable departure from Beer's Law but is a consequence of the inefficiency of the blue filters available (D. G. Anderson, personal communication). For instance, two kinds of filter tested here both had a noticeable transmission in the infrared, to which the photocell was somewhat sensitive and which was strongly emitted by the tungsten source.

Toxicity

Tests with a coagulase-negative staphylococcus (N.C.T.C. 2561) showed that the organism was able to grow in peptone water saturated with DDL and a dilution series showed that that growth was at least as good in DDL solution as in one containing an equal mol. prop. of formaldehyde. The dilution of reagent necessary for good growth was adequate for colour development, which agrees with figures given by Schales (1951) for the toxicity of acetylacetone to other bacteria.

Specificity

Owing to the mild conditions not many substances interfere, especially if the reaction time is kept to the minimum necessary for say 99% completion. The following have been tested.

Acetaldehyde gives rise to diacetyldihydrocollidine, with an absorption peak of similar intensity to that of DDL but at 388 mμ. instead of 412 mμ. The rate of formation is such that about 1% interference is to be expected from the same mol. prop. of acetaldehyde as formaldehyde. The reaction would not be suitable in the presence of large amounts of acetaldehyde.

Acetone, chloral, fural and glucose do not interfere. Ninhydrin probably does not if pure; one sample tested showed a small constant absorption equivalent to 0·005 mol. prop. of formaldehyde.

Amines can compete with ammonia in the Hantzsch reaction, though generally the rate of reaction is much less. It was found that 0·1 M-methylamine caused 4% loss, and 0·1 M-ethylenediamine 20% loss, of determined formaldehyde. Hexamethylenetetramine is gradually decomposed, yielding 80% of its formaldehyde on heating with reagent *A* for 2 hr. at 85°. Glycine does not interfere unless very concentrated.

Polymers of formaldehyde react slowly, probably by release of monomer. Paraformaldehyde gives a fairly rapid, but only partial, reaction. There appears to be at least one volatile polymer present in commercial 40% formalin which is completely unreactive, and which causes estimates of vapour concentration over formalin solutions to come out several times greater if measured by chromotropic acid, than with the present method. On the other hand, good agreement was obtained between the present method and Romijn's iodine method, as applied by Blair & Ledbury (1925) in similar estimations.

Periodate destroys the colour, but this can be prevented by the addition of excess iodine, followed immediately by excess thiosulphate.

Sulphites prevent the reaction in quite low concentration. 0·001 M sodium sulphite causes 90% loss of determined formaldehyde. No work has been done on the reactions involved.

Stability of colour

Strong oxidizing agents, particularly nitrous acid, destroy DDL either by complete breakdown of the molecule or by oxidation to the colourless diacetyllutidine. Daylight causes fading, probably aided by dissolved oxygen, but long exposure is necessary. In one test there was a 3% loss on standing in the dark in an open test tube overnight, which increased to 25% after standing all day in front of a window, but away from direct sunlight. A similar solution in a stoppered bottle showed no loss in the dark over 2 days.

EXPERIMENTAL

Preparation of diacetyldihydrolutidine and three related substances, and a comparison of their ultraviolet absorption

Acetylacetone, ammonia and formaldehyde are the simplest stable substances that can take part in a Hantzsch reaction. Because of this the reaction or its product might

Table 2. *Optical properties of cyclic compounds*

(For meaning of code names see Experimental section.)

Code name	Colour in daylight	Fluorescence (soft ultraviolet)	$\epsilon_{max.}$ ($\times 10^{-3}$)	λ ($\epsilon_{max.}$) (mμ.)
HH/Me	Strong-yellow	Yellow	7·7	412
HMe/Me	Pale-yellow-green	Pale-green	8·4	388
HH/OEt	Straw	Pale-green	6·6	370
HMe/OEt	None	Strong bluish white	7·8	353

Table 3. *Analyses of cyclic compounds*

(For meaning of code names see Experimental section.)

Code name	M.p. (°)	M.p. (literature) (°)	Carbon (%) Found	Carbon (%) Calculated	Hydrogen (%) Found	Hydrogen (%) Calculated
HH/Me	208 (decomp.)	198	67·7	68·4	7·78	7·72
HMe/Me	154	152–153	62·8	63·0	7·86	7·87
HH/OEt	178–180	174–176	62·1	61·7	7·50	7·51
HMe/OEt	129	127–129	69·0	69·6	8·04	7·86

be expected to show some exceptional properties, and to establish the colour reaction on a sound basis it was desirable to find out if this was so or not. It was known from recent work (Haley & Maitland, 1951) that acetaldehyde and ammonia react with both acetylacetone and acetoacetic ester under suitable mild conditions. In view of the DDL results and the general reactivity of formaldehyde, it was reasonable to suppose that ammonia, acetoacetic ester and formaldehyde would also react in a similar manner.

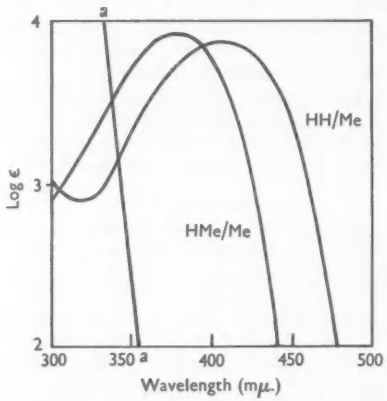


Fig. 5. Molecular absorption of 3:5-diacetyl-1:4-dihydrolutidine (HH/Me) and 3:5-diacetyl-1:4-dihydrocollidine (HMe/Me): *a–a* represents the cut-off by the generating solution.

It was therefore decided to prepare the end products of the four possible Hantzsch reactions between ammonia and formaldehyde, acetaldehyde, acetylacetone and acetoacetic ester. Preliminary experiments showed that reasonable yields were obtained in every case by using ammonium acetate as the only buffer for one-stage preparations in aqueous solution. It was also found that excess of ammonium salt was desirable, and the concentrations chosen for the preparations were: molar ammonium acetate, 0·2 M-β-diketone and 0·1 M-aldehyde. Solutions were made up as follows. Diacetyldihydrolutidine (HH/Me): 10 g. acetylacetone, 3·75 ml. 40% formalin; diacetyldihydrocollidine (HMe/Me): 10 g. acetylacetone, 3·05 g. acetaldehyde-ammonia; diethyl 1:4-dihydrolutidine-3:5-dicarboxylate (HH/OEt): 13 g. acetoacetic ester, 3·75 ml. 40% formalin; diethyl 1:4-dihydrocollidine-3:5-dicarboxylate (HMe/OEt):

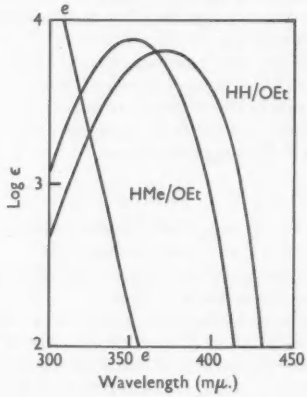


Fig. 6. Molecular absorption of diethyl 1:4-dihydrolutidine-3:5-dicarboxylate (HH/OEt) and diethyl 1:4-dihydrocollidine-3:5-dicarboxylate (HMe/OEt): *e–e* represents the cut-off by the generating solution.

13 g. acetoacetic ester, 3·05 g. acetaldehyde-ammonia. In addition, each solution contained 38 g. of ammonium acetate and was made up to 500 ml. The solutions were allowed to stand at 20–25° for 4 days and the precipitates filtered off and dried. Yields of crude product were: HH/Me, 6·7 g. or 77%; HMe/Me, 4·05 g. or 39%; HH/OEt, 9·0 g. or 71% and HMe/OEt, 11·1 g. or 83%.

The simple code names adopted refer to the different substituents in the basic ring system, HH referring to the ring methylene, HMe to the ring methyl-methylene, Me to the methyls of the acetyl groups and OEt to the ethoxy groups of the ester side chains. See also Figs. 5 and 6.

One recrystallization from ethanol was sufficient to give a product whose melting point agreed with that given in the literature, but up to three more were required before the colour of the crystals did not change further. There was also a marked increase in the fluorescence, particularly in the last compound (HMe/OEt). The melting points were also raised slightly, particularly the first compound (HH/Me, or DDL). These are given in Table 3, together with the analyses. As found by others (Hantzsch, Knoevenagel, quoted by Schiff & Prosio, 1895) there was a general deficiency in the carbon content. Repeat analyses on HH/Me gave figures for carbon of 66·7, 67·7 and 68·6% (averaged for Table 3), while two using a copper tube for combustion gave 69·4 and 69·7%.

Absorption spectra. It is clear from Figs. 5 and 6 that the compound HH/Me (DDL) is in no way exceptional. The strong-yellow colour is due to the absorption extending sufficiently far into the visible region; only the last compound, HMe/OEt, was finally obtained as completely colourless crystals, the two intermediate ones showing some absorption in the extreme blue. The optical properties are summarized in Table 2.

SUMMARY

A colour reaction for formaldehyde is described which depends on the synthesis of diacetyldihydrolutidine from acetylacetone and formaldehyde in the presence of excess of ammonium salt. The conditions are mild enough to allow of its use with living material, and its sensitivity and degree of specificity are comparable to those of other reactions requiring more severe conditions.

The work arose from investigations carried out on behalf of the Public Health Laboratory Service Committee on Formaldehyde Disinfection. I am indebted to Dr A. S. McFarlane and to Dr S. Jacobs, of the National Institute of Medical Research, for the use of a spectrophotometer, and to Dr J. Walker, also of the National Institute, for the microanalyses.

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Studies in the Biochemistry of Micro-organisms

90. ALTERNARIOL AND ALTERNARIOL MONOMETHYL ETHER, METABOLIC PRODUCTS OF *ALTERNARIA TENUIS*

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Although species of *Alternaria*, in the Dematiaceae family of moulds, are of common occurrence and of world-wide distribution, little work had been published until recently on their biochemical activities. Brian, Curtis, Hemming, Unwin & Wright (1949) reported that several strains of *A. solani* (E. and M.) Jones and Grout and one strain of *A. porri* (Ell.) Sacc. produce, in a variety of liquid media and in fair yields, a metabolic product which they named alternaric acid. This substance, which is not appreciably antibacterial, has a remarkably specific antifungal activity and is phytotoxic. The same workers and Jefferys (Brian *et al.* 1951) determined the optimum conditions for the production and isolation of alternaric acid and gave a more detailed account of its antifungal properties. They showed that while germination of the spores of some fungi, e.g. *Absidia glauca* and *Myrothecium verrucaria*, is prevented by 1 μg./ml. of alternaric acid, concentrations as high as 100 μg./ml. are required to prevent the germination of the spores of other fungi, e.g. *Botrytis allii*, although, with this fungus,

extension of the germ tubes is markedly retarded at a concentration as low as 0·01 μg./ml. Grove (1952) described alternaric acid, $C_{21}H_{30}O_8$, m.p. 138°, as a highly crystalline, colourless, optically inactive, unsaturated dibasic acid, giving a monomethyl ester, m.p. 106–107°.

Darpoux, Faivre-Amiot & Roux (1950) recorded the isolation of the following metabolic products from culture filtrates of a strain of *A. solani*: (*a*) a substance, which they named alternarine, colourless needles, m.p. 230°, which has marked antibacterial activity against a number of Gram+ and Gram− plant pathogenic bacteria; (*b*) an unnamed red pigment crystallizing in clusters of needles, which has no definite melting point but decomposes at about 260°; (*c*) a colourless crystalline substance, m.p. 136°, which they believe to be alternaric acid. They also isolated, by ether extraction of the dried mycelium of the same fungus: (*d*) an unnamed yellow crystalline substance which has no melting point but sublimes completely at 270° and dissolves immediately in alkalis giving an orange-red solution; (*e*) a red varnish which was not obtained crystalline. No analyses are recorded for any of these substances.

We have found that ethanolic extracts of the dried mycelium of two different strains of *A. tenuis*, which were isolated from tomatoes, give a strong purple colour with ethanolic ferric chloride. The present communication describes the isolation and study of the two substances responsible for this colour reaction. A comprehensive account of *A. tenuis* is given by Neergaard (1945) in a monograph on Danish species of *Alternaria* and *Stemphylium*. He points out that this species, which has an extraordinarily wide distribution and a great range of hosts, is usually found on the dead and weakened parts of plants on which it lives as a saprophyte. Infection experiments have shown, however, that it may appear as a weak parasite on a great many species of plants. Industrially, *A. tenuis* is also of considerable importance since, *inter alia*, it is the cause of serious losses in paper manufacture through its discoloration of wood pulp, and it is also the cause of the destruction of flax fibres because of its ability to decompose both pure pectin and pure cellulose.

The two strains of *A. tenuis*, L.S.H.T.M. catalogue numbers 94 and 430, were grown for a period of 5 weeks at 24° on Czapek-Dox solution. The washed, dried and ground mycelium was extracted with light petroleum to remove fatty materials. It was then redried and re-extracted with ether which removed completely the substances giving the purple ferric reaction. There separated from the ether a colourless crystalline solid, which, on fractionation from ethanol, gave two pure crystalline substances which we propose to name alternariol and alternariol monomethyl ether since neither of them is identical with alternaric acid or with the metabolic products of *A. solani* described by Darpoux *et al.* (1950).

The yields of the crystalline ether extract obtained presented points of some interest. With *A. tenuis* strain no. 94, when freshly isolated from nature, we obtained 8 g. of crystals from a batch of fifty flasks. On continued cultivation this yield steadily decreased until it had dropped to less than 0·1 g. per fifty flasks after 6 months. With strain no. 430 the first three batches of fifty flasks, after isolation from nature, yielded 8·3, 4·4 and 4·8 g. Sand cultures of this strain were then made and were preserved in evacuated and hermetically sealed tubes. Almost 18 months afterwards a fourth batch of fifty flasks, prepared from these sand cultures, gave the rather surprising yield of 18·0 g. The crystalline ether extracts, on fractionation from ethanol, gave about 10 times as much alternariol methyl ether as of alternariol itself.

Alternariol, $C_{14}H_{10}O_5$, m.p. 350° (decomp.), sublimes without decomposition at 250° in a high vacuum. It contains no methoxyl group. It does not dissolve in aqueous sodium bicarbonate but dissolves readily in cold aqueous sodium carbonate or sodium hydroxide giving a weak yellow-green solution which changes to orange-red on standing overnight. Its ethanolic solution, which gives no precipitate with Brady's reagent, gives an intense purple colour with ethanolic ferric chloride. It slowly reduces Fehling's solution on boiling and, in a parallel test with resorcinol, reduction occurs at approximately the same rate. On boiling a solution of it, in aqueous sodium hydroxide, with an equal volume of chloroform a bright cherry-red colour forms in the aqueous layer. Resorcinol gives an almost identical reaction, but catechol gives no significant colour. It dissolves in cold concentrated sulphuric acid giving a yellow solution with a strong blue-green fluorescence. It shows moderate antibacterial activity completely suppressing the growth of *Staphylococcus aureus* (Oxford H strain) at a concentration of 1:40000 and that of *Escherichia coli* at a concentration of 1:20000.

Alternariol methyl ether, $C_{15}H_{12}O_5$, m.p. 267° (decomp.), sublimes without decomposition at 180–200° in a high vacuum. It contains one methoxyl group, one methyl group attached to carbon and two active hydrogen atoms. It shows no optical activity. It does not dissolve in aqueous sodium bicarbonate, but is soluble in hot aqueous sodium carbonate. It readily dissolves in cold dilute aqueous sodium hydroxide giving a yellow-green solution which changes overnight to cherry-red. It gives the same reactions with ferric chloride, Fehling's solution, chloroform and sodium hydroxide and concentrated sulphuric acid as does alternariol. On demethylation, by heating at 140° with con-

centrated hydriodic acid, it gives an almost quantitative yield of alternariol.

The following functional derivatives of alternariol and alternariol methyl ether were prepared. (*a*) Diacetyl alternariol methyl ether, $C_{19}H_{16}O_7$, i.e. $C_{14}H_7O_2.(OCH_3)(O.CO.CH_3)_2$, by heating alternariol methyl ether with acetic anhydride and anhydrous sodium acetate. The product, m.p. 162–163°, is insoluble in cold aqueous 2N-sodium hydroxide, gives no ferric colour in ethanol and gives the same colour with concentrated sulphuric acid as does the parent substance. (*b*) Alternariol trimethyl ether, $C_{17}H_{16}O_5$, i.e. $C_{14}H_7O_2.(OCH_3)_3$, by long continued (14 days) methylation with ethereal diazomethane of alternariol methyl ether in dioxan solution or by methylation of alternariol or alternariol methyl ether with dimethyl sulphate and anhydrous potassium carbonate in acetone. The product, m.p. 162·5–164°, gives no colour with ethanolic ferric chloride and is insoluble in cold aqueous 2N-sodium hydroxide but dissolves slowly on boiling. (*c*) Hydrolysis product of alternariol trimethyl ether, $C_{17}H_{18}O_6$, i.e. $C_{14}H_9O_3.(OCH_3)_3$, by hydrolysis of alternariol trimethyl ether with aqueous N-sodium hydroxide in methanol solution followed by careful acidification. The product, m.p. 170–171°, dissolves in aqueous sodium bicarbonate and titrates in the cold as a monobasic acid. It gives no ferric colour in ethanol and does not reduce Fehling's solution. It is readily reconverted into alternariol trimethyl ether by sublimation in a high vacuum at its melting point. (*d*) Methyl ether methyl ester of (*c*), $C_{19}H_{22}O_6$, i.e. $C_{14}H_7O.(OCH_3)_5$, by methylation of alternariol methyl ether with dimethyl sulphate and aqueous potassium hydroxide. The ester, m.p. 124–125°, gives no ferric colour in ethanol and is insoluble in cold aqueous sodium hydroxide and only dissolves very slowly on boiling. It gives a purplish precipitate in a hydroxamic acid test, indicative of an ester grouping. (*e*) Methyl ether of (*c*), $C_{18}H_{20}O_6$, i.e. $C_{14}H_8O_2.(OCH_3)_4$, by alkaline hydrolysis in methanol of the ester (*d*). The free acid forms colourless microtablets, m.p. 181–182°. It titrates in the cold as a monobasic acid and dissolves readily in aqueous sodium bicarbonate. It gives no ferric reaction in ethanol and sublimes unchanged in a high vacuum at 170°.

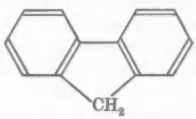
The general properties just described of the functional derivatives of alternariol and alternariol methyl ether justify the following conclusions as to the nature of the five oxygen atoms present in alternariol and in its methyl ether. Three of the oxygen atoms in alternariol, $C_{14}H_{10}O_5$, are probably phenolic in nature since they can be methylated with diazomethane. The formula for alternariol may therefore be written as $C_{14}H_7O_2.(OH)_3$. The insolubility of the diacetate (*a*) $C_{14}H_7O_2.(O.CO.CH_3)_2.(OCH_3)$, in cold aqueous alkali demonstrates the absence of a free carboxyl group in alternariol methyl ether and hence, inferentially, in alternariol itself. What then is the function of the two remaining oxygen atoms? We believe that they are present in a lactone ring since this hypothesis offers a simple explanation of the following experimentally established facts.

The alkaline hydrolysis product (*c*) of alternariol trimethyl ether contains one molecule of water more than its parent substance and is readily soluble in aqueous sodium bicarbonate, whereas the parent substance does not dissolve even in aqueous sodium hydroxide except on heating. These facts are most readily explained by postulating the opening of a lactone ring with the formation of a hydroxycarboxylic acid. Hence the formula $C_{17}H_{18}O_6$ for the hydrolysis product (*c*) may be expanded to $C_{13}H_7.(OCH_3)_3.(OH).(COOH)$, and the formula $C_{14}H_7O_2.(OCH_3)_3$ for alternariol trimethyl ether may be expanded to $C_{13}H_7.(OCH_3)_3.\begin{matrix}O\\|\\CO\end{matrix}$. The correctness of this hypothesis was established by methylating the hydrolysis product (*c*) when the methyl ether methyl ester (*d*), $C_{19}H_{22}O_6$, was obtained; this has therefore the expanded formula $C_{13}H_7.(OCH_3)_4.(COOCH_3)$. As would be expected, this substance is insoluble in cold aqueous sodium hydroxide but on hydrolysis with hot methanolic sodium hydroxide gives the free carboxylic acid (*e*), $C_{18}H_{20}O_6$, i.e. $C_{13}H_7.(OCH_3)_4.(COOH)$, which titrates sharply as a monobasic acid and is readily soluble in aqueous sodium bicarbonate.

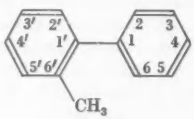
The presence of a $—COOCH_3$ group in the methyl ether methyl ester (*d*), $C_{19}H_{22}O_6$, was confirmed by heating it with concentrated hydriodic acid and red phosphorus. The substance was demethylated, one molecule of carbon dioxide was evolved and estimated and the decarboxylation product, $C_{13}H_{12}O_4$, was obtained as colourless rectangular platelets, m.p. 246–249°. The same substance was also formed in good yield when alternariol methyl ether was fused with potassium hydroxide at 300°, although, when alternariol methyl ether was heated with concentrated hydriodic acid and red phosphorus no carbon dioxide was evolved and alternariol was isolated in good yield. The decarboxylation product gives a purple-blue ferric reaction in aqueous ethanolic solution. It is insoluble in aqueous sodium bicarbonate but dissolves in aqueous sodium carbonate and in aqueous sodium hydroxide. It contains one methyl group attached to carbon. On methylation with dimethyl sulphate and anhydrous potassium carbonate in dry acetone it forms a tetramethyl ether, $C_{17}H_{20}O_4$, i.e. $C_{13}H_8.(OCH_3)_4$, as colourless irregular tablets, m.p. 73–74°, which give

no ferric colour and which do not dissolve in aqueous sodium hydroxide even on boiling.

The parent hydrocarbon corresponding to the decarboxylation product $C_{13}H_{12}O_4$ is thus clearly $C_{13}H_{12}$ and, since the decarboxylation product is known to contain a C-CH_3 group, the formula for the parent hydrocarbon may be written $C_{12}H_9.CH_3$. The same conclusion is arrived at from consideration of the formula $C_{12}H_7.(OCH_3)_4.(COOCH_3)$ for the methyl ether methyl ester (*d*).



(I)



(II)

A plausible structure for the parent hydrocarbon $C_{12}H_9.CH_3$ was deduced from the following facts. (i) Alternariol methyl ether and the methyl ether methyl ester (*d*), $C_{19}H_{22}O_6$, resisted all attempts to reduce them catalytically. Further, the methyl ether methyl ester (*d*) resisted an attempt to ozonolyse it. Hence we may conclude that neither alternariol methyl ether nor the methyl ether methyl ester (*d*) nor the parent hydrocarbon $C_{13}H_{12}$ contains an ethylenic double bond. (ii) A mixture of alternariol methyl ether and electrolytic zinc dust was subjected to distillation in a current of hydrogen. Fluorene ($C_{13}H_{10}$, I) was isolated from the mixture of volatile degradation products obtained and was conclusively identified by analysis and by comparison with an authentic specimen including X-ray powder examination.

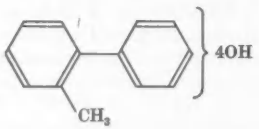
The simplest structure for the parent hydrocarbon $C_{13}H_{12}$ which satisfies the following requirements (*a*) contains a C-CH_3 group; (*b*) contains no ethylenic double bonds; and (*c*) gives fluorene on zinc-dust distillation, is II.

The fact that an unsubstituted fluorene is obtained by zinc-dust distillation indicates that the C-CH_3 group in the parent hydrocarbon $C_{13}H_{12}$ is probably in the ortho position, i.e. 6 or 6', since if it were in any other position a methylfluorene would be expected to arise by zinc-dust distillation.

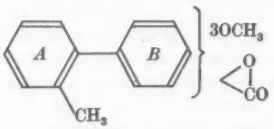
Accepting this hypothesis, partial structure III may be assigned to the decarboxylation product $C_{13}H_{12}O_4$ and partial structure IV to alternariol trimethyl ether.

Evidence as to the positions occupied by the three methoxyl groups and the lactone grouping in alternariol trimethyl ether was obtained as follows. A solution of the hydrolysis product (*c*) of alternariol trimethyl ether in cold aqueous N sodium hydroxide was oxidized with potassium permanganate at a temperature not exceeding 35°. The two pure oxidation products isolated were identified as 3:5-dimethoxyphthalic acid (V) and the previously unknown 2-carboxy-3:5-dimethoxyphenylglyoxylic acid (VI). The structure (VI) of the second oxidation product was established as follows. The product, $C_{11}H_{10}O_7$, H_2O, m.p. 173–174°, contains two methoxyl groups and titrates sharply in the cold as a dibasic acid. It is readily soluble in cold aqueous sodium bicarbonate. On oxidizing the product in the cold with alkaline hydrogen peroxide, 3:5-dimethoxyphthalic acid (V), $C_{10}H_{10}O_6$, was formed in good yield. On heating the product with concentrated hydriodic acid and red phosphorus it was demethylated, one molecule of carbon dioxide was evolved and was estimated and 3:5-dihydroxyphenylacetic acid (VII) was isolated. The identity of the 3:5-dihydroxyphenylacetic acid, m.p. 130–131°, was established by comparison (as acid and as the previously unknown methyl ester) with authentic synthetic specimens.

The isolation of (VI) fixes the structure of ring *B* in structure IV which may now be expanded to VIII. This structure is supported by the fact that alternariol itself gives an intense purple salicylic ferric colour so that one of the hydroxyl groups in alternariol is almost certainly in the ortho position to the potential carboxyl group.



(III)



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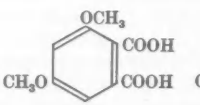

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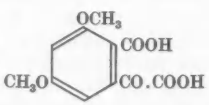

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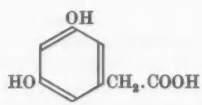
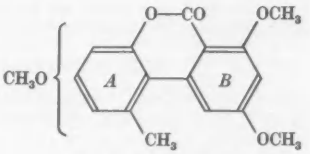


(VII)

The structure of the alkaline hydrolysis product (*c*), $C_{17}H_{18}O_6$, of alternariol trimethyl ether then becomes IX. This compound (IX), but not its methyl ether, gives a positive Gibbs reaction (1927). Hence position 5′ in ring *A* is probably unsubstituted and, if so, the OCH_3 group in ring *A* must be either in position 3′ or 4′.

The general correctness of these deductions and the fact that alternariol trimethyl ether has probably structure X and not the alternative structure with the CH_3O group in position 3′ was proved by its synthesis.



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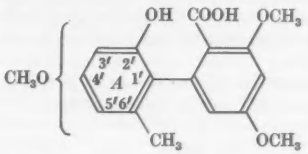
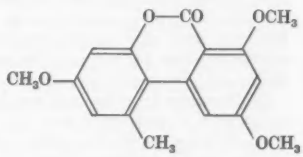
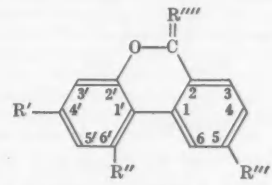


(IX)

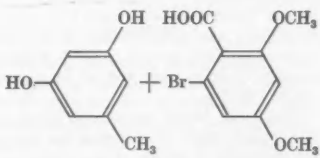


(X)

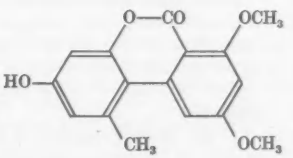
Alternariol trimethyl ether



(XI)



(XII) (XIII)



(XIV)

Hurtley (1929) condensed resorcinol with *o*-bromobenzoic acid in an alkaline medium with copper sulphate as catalyst and obtained what has since been shown to be 4′-hydroxydibenzo-α-pyrone (XI, R′ = OH, R″ = R‴ = H, R′′′′ = > O).

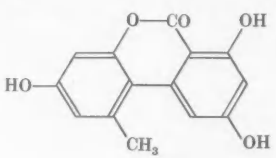
This reaction has proved to be of general application in the synthesis of substituted dibenzo-α-pyrones. It was extensively studied and used by Adams and his collaborators in their synthesis of cannabinol (XI, R′′′′ = :$(Me)_2$, R‴ = Me, R″ = OH, R′ = *n*-C_5H_{11}) and substances related to cannabinol. Cannabinol is a constituent of Egyptian and Indian hashish which is obtained from *Cannabis indica*. During the course of this work they synthesized 6′-*n*-amyl-4′-hydroxy-5-methyldibenzo-α-pyrone by condensing olivetol (1-*n*-amyl-3:5-dihydroxybenzene) with 2-bromo-4-methylbenzoic acid (Adams, Pease, Clark & Baker, 1940*a*; Adams, Baker & Wearn, 1940*b*). The constitution of the pyrone was rigorously proved.

In a number of analogous syntheses orcinol (3:5-dihydroxy-1-methylbenzene) was condensed with different substituted *o*-bromobenzoic acids. Thus Adams *et al.* (1940*a*) obtained 4′-hydroxy-6′-methyldibenzo-α-pyrone from orcinol and *o*-bromobenzoic acid, and 4′-hydroxy-5:6′-dimethyldibenzo-α-pyrone from orcinol and 2-bromo-4-methylbenzoic acid. Lederer & Polonsky (1948), in a study of the synthesis of 4:4′-dihydroxydibenzo-α-pyrone which Lederer (1942) had isolated from castoreum, the secretion of the scent glands of the beaver (*Castor fiber*), condensed orcinol with 2-bromo-5-methoxybenzoic acid and obtained 4′-hydroxy-4-methoxy-6′-methyldibenzo-α-pyrone. All these findings point to the conclusion that, when substituted *o*-bromobenzoic acids are condensed in the Hurtley reaction with orcinol, condensation takes place at the carbon atom in the orcinol nucleus which is ortho and para to the two hydroxyl groups.

In an analogous manner we used the Hurtley reaction to condense orcinol (XII) with 2-bromo-

4:6-dimethoxybenzoic acid, m.p. 151–152° (XIII), which was synthesized from 2-amino-4:6-dimethoxytoluene (Curd & Robertson, 1933) via the Sandmeyer reaction followed by permanganate oxidation of the methyl group to carboxyl. The resulting condensation product, 4′-hydroxy-3:5-dimethoxy-6′-methyldibenzo-α-pyrone (XIV), m.p. 293–296°, was methylated giving 3:4′:5-trimethoxy-6′-methyldibenzo-α-pyrone (X), m.p. 162·5–163°, not depressed on admixture with alternariol trimethyl ether, m.p. 162·5–164°. Both specimens could also be obtained in a form melting at a lower temperature (about 140°), and the yellow solution of each specimen in cold concentrated sulphuric acid showed a strong blue-green fluorescence in ultraviolet light.

It follows, therefore, that alternariol is 3:4′:5-trihydroxy-6′-methyldibenzo-α-pyrone (XV).



(XV)
Alternariol.

Accepting structure XV for alternariol the designations of the different methyl derivatives and partial degradation products of alternariol follow. Thus the hydrolysis product of alternariol trimethyl ether becomes 2′-hydroxy-3:4′:5-trimethoxy-6′-methyldiphenyl-2-carboxylic acid (*c*) p. 428, and the decarboxylation product of the methyl ether methyl ester (*d*) and the potash fusion product of alternariol methyl ether becomes 2′:3:4′:5-tetrahydroxy-6′-methyldiphenyl.

The exact position of the methoxyl group in the second metabolic product of *A. tenuis*, alternariol monomethyl ether, is uncertain though it is believed to be in either position 4′ or 5 for the following reasons. (i) Alternariol monomethyl ether, like alternariol itself, gives an intense purple salicylic ferric colour. Hence we believe that position 3 in alternariol monomethyl ether is occupied by a hydroxyl and not by a methoxyl group. (ii) In the Gibbs test (1927) alternariol gives a strong purple colour in 1 min.; after the same time alternariol monomethyl ether gives a similar but paler purple colour. This is readily understood if the methoxyl group occupies either position 4′ or 5 but not if it occupies position 3. Hence we believe that the metabolic product, alternariol monomethyl ether, is 3:(4′ or 5)-dihydroxy-(5 or 4′)-methoxy-6′-methyldibenzo-α-pyrone. It is of interest here to note that both orcinol and resorcinol give a strong purple colour in the Gibbs test, though most other phenols, including phenol itself, give a strong pure-blue colour.

Although a number of substituted dibenzo-α-pyrones of both plant and animal origin have been described we believe that alternariol and its monomethyl ether are the first recorded substituted dibenzo-α-pyrones of fungal origin.

EXPERIMENTAL

All melting points are uncorrected except where otherwise stated. Methoxyl estimations and determination of titration equivalents were carried out in this Department, all other estimations by Weiler and Strauss, Oxford.

History of cultures

We are indebted to Mr G. Smith of this Department for the following mycological notes on the cultures used in this investigation.

Both the strains L.S.H.T.M. catalogue numbers 94 and 430 were isolated here by Mrs S. Marcus from tomato. In both cases the first cultures produced spores abundantly, but, whilst no. 430 is still in good condition and sporing freely on most culture media, no. 94 has deteriorated and produces spores only sparsely, and on some media not at all. However, when first isolated, the two strains were very similar, in colour and texture of colonies, in length of spore chains and in shape and size of spores.

Colonies spread rapidly on all media; on potato agar fairly dense, with a basal felt of dark mycelium overgrown with dirty-white to greyish funiculose hyphae; on Czapek agar similar; on corn-meal agar thin, transparent, very dark dull green to almost black, sporing well; spore chains mostly long, often branched, with up to 10 or 11 spores; spores extremely polymorphous, ovate, conical, cylindrical or irregular, mostly beakless or with very short beak but occasionally with fairly long definite beak, with or without longitudinal septa, rough or smooth, mostly fairly deep olive-brown, number of cross septa 1–9, of longitudinal septa 0–4, size 15–50 × 8–17 μ.

The most recent taxonomic study of *Alternaria* is by Neergaard (1945). He divides the genus into three groups on the basis of length of spore chains. The species to be considered in the present connexion are the three species in the group Longicatenatae, *A. tenuis*, *A. circinans* and *A. resedae*, and also *A. tenuissima*, in the group Brevicatenatae, which is stated to produce long chains in artificial culture. The spores of *A. circinans* and *A. resedae* are somewhat similar, being comparatively uniform in shape and mostly lacking longitudinal septa. *A. tenuis* is sharply distinguished from these by its markedly polymorphous spores, mostly with longitudinal septa. *A. tenuissima* differs from *A. tenuis* in the somewhat greater size of the spores, in the spores mostly having fairly long beaks, and in the lesser degree of variation in shape and size. There is little doubt that the two isolates nos. 94 and 430 are to be assigned to *A. tenuis* auct. sensu stricto in Neergaard's sense. Neergaard states that this species is a ubiquitous saprophyte and weak polyphagous parasite. Although he does not state categorically that it attacks tomato, one of the isolates on which he based his study, and assigned by him to *A. tenuis* sensu stricto, was obtained from tomato.

Table 1. *Cultural characteristics of* Alternaria tenuis *strain no. L.S.H.T.M.* 94

Incubation period (days)	pH of culture filtrate	Residual glucose by polarimeter (%)	Culture filtrate ether extract/flask: Total extract (g.)	Culture filtrate ether extract/flask: Crystalline extract (mg.)	Mycelium wt./flask (g.)	Mycelium ether extract/flask: Total extract (g.)	Mycelium ether extract/flask: Crystalline extract (mg.)
21	7·3	1·24	—	—	3·19	0·27	69
25	7·3	0·78	0·23	37	3·82	0·33	81
31	7·3	0·56	0·24	40	3·99	0·41	161
39	7·5	0·09	0·28	54	4·11	0·44	160

Cultural conditions

Batches of a hundred 1 l. conical flasks, plugged with cotton wool, were prepared, each containing 350 ml. of Czapek-Dox solution, i.e. glucose, 50·0 g.; $NaNO_3$, 2·0 g.; KH_2PO_4, 1·0 g.; KCl, 0·5 g.; $MgSO_4$, $7H_2O$, 0·5 g.; $FeSO_4$, $7H_2O$, 0·01 g.; distilled water, 1 l. After sterilization, each flask was inoculated with spore suspensions obtained from freshly grown Czapek-agar slopes of the chosen strain of *A. tenuis*, either no. 94 or no. 430, and incubated in the dark at 24°. Growth of the mould was rather slow. The mycelium was a dirty-grey colour and contained patches of dull-olive green and black with a black reverse. It was normally very slimy to the touch. The culture fluid became pale brown in colour towards the end of the incubation period.

In order to determine the optimum time for harvesting, batches of fifty flasks were harvested after 21, 25, 31 and 39 days' incubation. Determinations were made of pH (colorimetrically), residual glucose (polarimetrically) in the culture filtrates, weight of mycelium (dried to constant weight), and of ether extract from the mycelium and from the culture filtrate as described below. The results obtained are shown in Table 1.

Isolation and purification of alternariol and alternariol methyl ether

Batches of fifty flasks were harvested after 35 days. The pale-brown culture fluid was decanted from the mould mycelium which was scraped out and squeezed in butter muslin. The mycelium was then thoroughly washed with water, strained again through muslin and pressed as dry as possible in a tincture press. It was thoroughly dried in a vacuum oven at 45–50° and ground to a fine powder in a coffee mill. The average weight of dry mycelium from a batch of fifty flasks of either strain of *A. tenuis* was 200–210 g.

The dried, ground mycelium was extracted in Soxhlets with light petroleum (b.p. 40–60°) for 8 hr. The resulting light-orange-yellow extract, on removal of the solvent, gave a yellow oil containing a small quantity of a colourless wax-like solid. This extract gave no ferric reaction in ethanolic solution.

The mycelium was now re-dried and was then re-extracted with dry ether for 12–13 hr. when extraction was practically complete. Within 20 min. of commencing the extraction a colourless crystalline solid began to separate. The majority of the product was extracted in the first hour. After the extraction was complete the separated solid was collected by filtration; the orange-pink ethereal filtrate was concentrated and yielded initially a further crop of crystals and finally a red oily liquid.

In the case of *A. tenuis* strain no. 94 the crystals which separated during the extraction process formed 80–90% of the total (8 g.) crystalline extract and consisted of almost pure *alternariol methyl ether* melting only 4° below the m.p. of the pure substance, i.e. 267° (decomp.). The crystals which were obtained on concentration of the ethereal mother liquor consisted of a mixture of alternariol methyl ether and *alternariol*, which, when purified, melted at 350° (decomp.).

With *A. tenuis* strain no. 430 the crystals which separated during the extraction process consisted of a mixture of alternariol methyl ether and alternariol, which on heating softened at approx. 260° and decomposed completely at 330–340°. The combined yields of crystalline solid from the first four batches of fifty flasks of strain no. 430 were 8·3, 4·4, 4·8 and 18·0 g. (see p. 422). The proportion of alternariol methyl ether to alternariol was approximately 10:1 with strain no. 94 and was slightly less with strain no. 430.

Mixtures of alternariol methyl ether and alternariol can be readily separated into their components by means of the much higher solubility of the latter compound in ethanol. Thus a hot ethanolic solution of a mixture of the two substances, on cooling, deposits alternariol methyl ether in the form of colourless feathery needles. Alternariol remains in solution in the ethanol from which it may be separated, by diluting with hot water, in the form of colourless needles which are finally purified by sublimation in a high vacuum at 250°. Alternariol methyl ether crystallizes readily from ethanol or dioxan, the latter solvent being more suitable for larger quantities. The approximate solubilities in these solvents at their boiling points and at 0–5° are, in ethanol 0·6 and 0·2%, and in dioxan 5·0 and 1·4% respectively. The product isolated from some batches of mycelium, particularly the early batches of strain no. 94, was accompanied by a pink contaminant which persisted throughout the above purification processes.

General properties of alternariol

Alternariol crystallizes from aqueous ethanol in colourless needles, m.p. 350° with decomposition and with the formation of a small microcrystalline sublimate of unchanged material. It sublimes without decomposition in a high vacuum at 250°. It contains no N, S, Cl or methoxyl. (Found: C, 65·4; H, 3·9. $C_{14}H_{10}O_5$ requires C, 65·1; H, 3·9%.) Alternariol is more soluble than its methyl ether in ethanol, methanol, acetone and ether and almost insoluble in benzene or light petroleum. It does not dissolve in water or in aqueous $NaHCO_3$, but readily dissolves in cold aqueous Na_2CO_3 or NaOH giving a weak yellow-green solution which changes to an orange-red colour on standing overnight. Its ethanolic solution gives no precipitate on the addition of an

equal volume of Brady's reagent (0·32% 2:4-dinitrophenylhydrazine in aqueous 2N-HCl). It gives the following reactions. (*a*) Its ethanolic solution gives an intense purple colour with ethanolic $FeCl_3$; (*b*) on boiling with a mixture of equal volumes of $CHCl_3$ and aqueous NaOH, a bright cherry-red colour develops in the alkali layer; resorcinol gives an almost identical reaction but catechol gives no significant colour; (*c*) reduces hot Fehling's solution at about the same rate as resorcinol; (*d*) dissolves in cold conc. H_2SO_4 to a yellow solution with a strong blue-green fluorescence.

General properties of alternariol methyl ether

Alternariol methyl ether crystallizes from ethanol or dioxan in fine colourless needles, m.p. 267° (decomp.). It sublimes without decomposition in a high vacuum at 180–200°. It contains no N, S or Cl but contains one methoxyl group. (Found: C, 66·1, 66·3; H, 4·4, 4·5; OCH_3, 11·0, 11·4; *C*-CH_3, 7·5 = 1·36 *C*-CH_3 groups; active H atoms (in pyridine), 1·67; mol.wt. (Rast), 294. $C_{15}H_{12}O_5$ requires C, 66·2; H, 4·4; 1OCH_3, 11·4; 1*C*-CH_3, 5·5%; mol.wt. 272.) A saturated ethanolic solution (approx. 0·3%) did not exhibit any optical activity when viewed in a 2 dm. tube. It is moderately soluble in dioxan, ethanol and acetone, slightly soluble in ether and almost insoluble in benzene and light petroleum. It does not dissolve in water or in aqueous $NaHCO_3$ but dissolves in hot aqueous Na_2CO_3, although on cooling a microcrystalline deposit separates. It readily dissolves in cold dilute aqueous NaOH giving a yellow-green solution which changes overnight to cherry red. It gave the same reactions (*a*), (*b*), (*c*) and (*d*) as are described above for alternariol.

Demethylation of alternariol methyl ether. Isolation of alternariol

In a Zeisel estimation alternariol methyl ether (0·0981 g.) was refluxed for 4 hr. at 140° with constant b.p. HI (15 ml.). The CH_3I formed was led over in CO_2 into ethanolic $AgNO_3$. (Found: OCH_3, 11·9%.) On cooling, the HI solution contained a solid which was collected, washed with hot water and dried; 0·0874 g. = 94% of the theoretical; colourless microneedles. The product was purified by sublimation in a high vacuum at 250° giving a colourless sublimate, m.p. 350° (decomp.) not depressed on admixture with alternariol from *A. tenuis*. (Found: C, 65·3; H, 3·9. $C_{14}H_{10}O_5$ requires C, 65·1; H, 3·9%.)

Derivatives of alternariol and alternariol methyl ether

Acetylation of alternariol methyl ether. Alternariol methyl ether was boiled with anhydrous sodium acetate and acetic anhydride for 5 min. and the cooled mixture was diluted with water. *Diacetylalternariol methyl ether* after three crystallizations from ethanol formed long colourless needles, m.p. 162–163°. (Found: C, 63·9; H, 4·4; OCH_3, 8·7. $C_{19}H_{16}O_7$, i.e. $C_{15}H_{10}O_3.(O.CO.CH_3)_2$, requires C, 64·0; H, 4·5; 1OCH_3, 8·7%.) It is insoluble in cold aqueous 2N-NaOH. Its ethanolic solution gives no colour with ethanolic $FeCl_3$. It gives the same colour with conc. H_2SO_4 as does the parent substance.

Methylation of alternariol methyl ether and alternariol.

With dimethyl sulphate and K_2CO_3 in acetone. Alternariol methyl ether (1 g.), anhydrous K_2CO_3 (4 g.), dimethyl sulphate (4 ml.) and dry acetone (100 ml.) were heated under reflux for 7 hr. The solution, which no longer gave a purple ferric colour, was filtered and the residue washed with fresh acetone. The pale-yellow filtrate, on concentration, gave clusters of colourless feathery needles (0·95 g.) which on recrystallization from acetone gave *alternariol trimethyl ether* as colourless feathery needles, m.p. 162·5–164° (corr.), resetting and then remelting at 162·5–163·5°. (Found: C, 67·9; H, 5·2; OCH_3, 30·5, 30·7. $C_{17}H_{16}O_5$ requires C, 68·1; H, 5·4; 3OCH_3, 31·0%.) It is insoluble in cold aqueous 2N-NaOH but dissolves slowly on boiling. It gives no colour with ethanolic $FeCl_3$. It gives the same colour as the parent substance with conc. H_2SO_4. On boiling with a mixture of equal volumes of $CHCl_3$ and aqueous NaOH, the red colour given under these conditions by alternariol and by its methyl ether is no longer produced. Further, resorcinol dimethyl ether yielded on similar treatment only a light-orange colour in striking contrast to the intense red colour given by resorcinol itself.

Alternariol similarly treated gave an identical product.

With diazomethane. An ethereal solution of diazomethane was added to a solution of alternariol methyl ether (0·10 g.) in dioxan (10 ml.). Methylation took place very slowly, and it was necessary to continue the reaction for 14 days with periodic addition of fresh diazomethane to ensure full methylation. On removal of the solvents there remained a mobile gum (0·09 g.) which crystallized on the addition of ether. Recrystallization from acetone gave colourless feathery needles, m.p. 162·5–164°, not depressed on admixture with the trimethyl ether as prepared above.

Alkaline hydrolysis of alternariol trimethyl ether

Isolation of 2′-hydroxy-3:4′:5-trimethoxy-6′-methyldiphenyl-2-carboxylic acid. A solution of alternariol trimethyl ether (0·2006 g., m.p. 162·5–164°) in methanol (40 ml.) and N-NaOH (2·00 ml.) was refluxed for 4·5 hr. in an atmosphere of O_2- and CO_2-free N_2. After cooling, the colourless solution was titrated against N-HCl using phenolphthalein as indicator. (Found, equivalent, corrected for blank: 299. $C_{17}H_{16}O_5$ titrating as a monobasic acid requires 300.) The neutralized solution was concentrated to about 0·25 vol., cooled, acidified to Congo red with N-HCl, and the colourless crystalline solid which separated was collected, washed with water and dried. The *product* (0·195 g.) consisted of heavy colourless tablets, m.p. 170–171°. (Found: C, 63·3; H, 5·8; equiv. by titration, 316. $C_{17}H_{18}O_6$ requires C, 64·1; H, 5·7%; equiv. titrating as a monobasic acid, 318.) The product is insoluble in water but dissolves in aqueous $NaHCO_3$. With conc. H_2SO_4 it gives a weak-yellow solution with a slight green fluorescence. Both the colour and the fluorescence are much less intense than those given by alternariol trimethyl ether but both become more intense on heating. It does not reduce Fehling's solution and its ethanolic solution gives no colour with ethanolic $FeCl_3$. The product could not be purified by recrystallization since all such attempts yielded a product of slightly lower melting point which was not completely soluble in cold aqueous 2N-NaOH. The m.p. 170–171° was depressed on admixture with alternariol trimethyl ether, m.p. 162·5–164°. The acid was readily reconverted into alternariol trimethyl ether by sublimation at its melting point in a high vacuum and crystallization of the colourless transparent sublimate from acetone.

Methylation of alternariol methyl ether with dimethyl sulphate and aqueous KOH

Isolation of the methyl ester of 2′:3:4′:5-tetramethoxy-6′-methyldiphenyl-2-carboxylic acid. A solution of alternariol methyl ether (0·50 g.) in methanol (10 ml.) was refluxed and dimethyl sulphate (10 ml.) and 20% aqueous KOH (40 ml.) were added dropwise over a period of 20 min., the KOH being added at such a rate as to keep the mixture alkaline; the mixture was then refluxed for 2·75 hr. The opalescent yellow solution, pH approx. 8·0, on cooling deposited a sticky, pale-brown, gum which was collected, washed with water and dried. The gum (0·31 g.), which readily crystallized on trituration with methanol, on recrystallization from methanol, yielded the *ester* as colourless heavy prisms, m.p. 124–125°. (Found: C, 66·1; H, 6·3; OCH_3, 44·3. $C_{18}H_{22}O_6$ requires C, 65·9; H, 6·4; 5OCH_3, 44·8%.) The ester is insoluble in cold aqueous NaOH and only dissolves very slowly on boiling. It gives no ferric colour in ethanolic solution. In conc. H_2SO_4 it gives a weak yellow colour with a slight green fluorescence, both colours being far less intense than those given by alternariol methyl ether. In a hydroxamic acid test it gives a purplish precipitate indicative of an ester grouping (Feigl, 1939).

The methylation solution filtrate from the above crude ester was acidified to Congo red. The resulting gummy deposit was collected, washed with water and dried (89 mg.). It was crystallized from aqueous methanol giving flat, colourless microtablets (wt. 56 mg.), m.p. 179°, not depressed on admixture with the acid $C_{18}H_{20}O_6$ obtained by alkaline hydrolysis of the ester $C_{19}H_{22}O_6$ (see following paragraph).

Dilutions (×1000)	1:20	1:40	1:80	1:160	1:320	Control
Staph. aureus	−	−	+	+ +	+ +	+ +
Esch. coli	−	+ +	+ +	+ +	+ +	+ +

Alkaline hydrolysis of the ester $C_{19}H_{22}O_6$

Isolation of 2′:3:4′:5-tetramethoxy-6′-methyldiphenyl-2-carboxylic acid. A mixture of the ester (0·20 g.), aqueous 2N-NaOH (20 ml.) and methanol (2 ml.) was refluxed for 3 hr. A slight precipitate was separated by filtration and the cooled filtrate was acidified to Congo red dropwise with conc. HCl, when a colourless solid (0·15 g.) separated. This substance crystallized from aqueous methanol giving the *hydrolysis product* as colourless microtablets, m.p. 181–182°. (Found: C, 64·8; H, 6·2; OCH_3, 37·7; equiv. by titration, 329. $C_{18}H_{20}O_6$ requires C, 65·1; H, 6·1; 4OCH_3, 37·4%; equiv. titrating as a monobasic acid, 332.) The hydrolysis product sublimes unchanged in a high vacuum at 170°. It is insoluble in water but readily dissolves in aqueous $NaHCO_3$. It does not give a ferric reaction in ethanolic solution and conc. H_2SO_4 produces only a pale-yellow solution with a slight green fluorescence but this colour becomes more intense on heating the solution.

Colour reactions with 2:6-*dichloroquinone chloroimide* (*Gibbs*, 1927)

The reaction was carried out as follows. About 1 mg. of the substance under test was dissolved in 1 ml. of sodium borate buffer, pH 9·2, by heating if necessary. To each solution, after cooling, was added one drop of a freshly prepared aqueous suspension of 2:6-dichloroquinone chloroimide. The following colour reactions were obtained: natural alternariol, immediate strong purple reaching maximum after 1 min.; natural alternariol monomethyl ether, immediate paler purple; synthetic alternariol dimethyl ether (see p. 432) and natural alternariol trimethyl ether (in acetone solution), negative even after 2 hr.; alkaline hydrolysis product, $C_{17}H_{18}O_6$, of alternariol trimethyl ether, i.e. 2′-hydroxy-3:4′:5-trimethoxy-6′-methyldiphenyl-2-carboxylic acid (see p. 428), immediate deep blue reaching maximum after 1 min.; 2′-methyl ether, $C_{18}H_{20}O_6$, of the above, i.e. 2′:3:4′:5-tetramethoxy-6′-methyldiphenyl-2-carboxylic acid (see previous para.), negative; orcinol or resorcinol, deep purple; phenol, deep blue.

Antibacterial activity of alternariol (by Mrs S. Marcus)

Alternariol is insoluble in water but a dilute solution was obtained in phosphate buffer pH 8·0. A 1:10000 solution of alternariol in phosphate buffer pH 8·0 (4·5 ml.) was mixed with an equal volume of sterile glucose broth. Half of the mixture (4·5 ml.) was transferred to a second tube containing glucose broth (4·5 ml.) and so on, giving a series of solutions of concentrations of alternariol of 1:20 000, 1:40 000, 1:80000, 1:160000 and 1:320000. One batch of such tubes was inoculated with a 24 hr. culture of the Oxford H strain of *Staphylococcus aureus* and the other batch with a similar culture of *Escherichia coli* N.C.T.C. strain no. 86. The tubes were incubated for 24 hr. at 37° with the following results (−, no growth; +, growth; + +, growth as in control).

A series of controls using only phosphate buffer, pH 8·0, showed no growth inhibition.

Oxidation of alternariol trimethyl ether with alkaline $KMnO_4$. *Isolation of* 3:5-*dimethoxyphthalic acid and* 2-*carboxy*-3:5-*dimethoxyphenylglyoxylic acid*

Alternariol trimethyl ether (2 g.) was refluxed with N-NaOH (80 ml.) in N_2 for 0·5 hr. The resulting colourless solution was cooled, and a solution of $KMnO_4$ (7 g.) in water (100 ml.) was added with stirring and cooling. The temperature of the reaction solution rose to a maximum of 35°. After 24 hr. at room temperature, the heavy MnO_2 precipitate was separated by filtration and washed with water. The alkaline filtrate and washings were combined and adjusted to pH 7 with 2N-H_2SO_4. The resulting solution was evaporated *in vacuo* at 35° until approximately 20 ml. of distillate had been collected, the vapours being passed through Brady's reagent in which, however, no precipitate was formed. The neutral, partially evaporated, oxidation solution was acidified to Congo red and extracted with ether (4 × 1 vol.). On removal of the solvent there remained a yellow gum (0·69 g.) which, on trituration with dry ether, crystallized in very pale-yellow rectangular plates. Recrystallization from hot water gave oxidation product I (0·48 g.) which on further recrystallization melted at 184° (decomp.).

The ether-extracted oxidation solution was adjusted to pH 7·0 with aqueous NaOH and evaporated *in vacuo* at 35–40° to 30 ml. The evaporated solution was re-acidified and extracted with ether (5 × 1 vol.). Removal of the solvent

gave a pale-yellow gum (0·39 g.) containing colourless needles. The oxidation solution was now extracted continuously with ether for three successive 10 hr. periods yielding extracts weighing 0·35, 0·07 and 0·04 g., respectively. The gum (0·39 g.) obtained above by hand extraction with ether and the first of the continuous ether extracts (0·35 g.) which contained some gum but was largely crystalline were washed with dry ether to remove the gum. The combined crystals were purified by crystallization from water giving oxidation product II as heavy colourless needles, m.p. 173–174° (decomp.).

General properties of oxidation product I. Identification as 3:5-dimethoxyphthalic acid (*V*). Oxidation product I crystallizes from water in cream-coloured rectangular prisms, which on heating melt at 184° to a colourless liquid which on cooling resets to colourless needles, m.p. 148°. When heated to 100° in a high vacuum, oxidation product I loses water of crystallization (7·34%) without change in melting point. This loss in weight, if due to the removal of 1 mol. water, corresponds to the formation of a compound of mol.wt. 228. (Found, on above dried material: C, 53·3; H, 4·4; OCH_3, 27·0; equiv. by titration, 109. Calc. for $C_{10}H_{10}O_6$: C, 53·1; H, 4·4; $2OCH_3$, 27·4%; mol.wt. 226; equiv. titrating as a dibasic acid, 113.) The acid gives a wine-red colour on heating alone with conc. H_2SO_4. On heating the acid with resorcinol and conc. H_2SO_4, followed by dilution with water and addition of excess NaOH, there is obtained a yellow solution with an intense green fluorescence. Both these reactions are given by 3:5-dimethoxyphthalic acid.

The unusual behaviour on heating of 3:5-dimethoxyphthalic acid, which has been noted in detail by Oxford & Raistrick (1933, p. 650), renders it unsuitable for diagnostic purposes. For this reason the anhydride and dimethyl ester were prepared for the purpose of comparison.

The acid was heated at 190° in an oil bath for 5 min., by which time all effervescence had ceased. On cooling, the melt set to a mass of colourless needles which were purified either by crystallization from *o*-xylene or by sublimation in a high vacuum giving long colourless needles, m.p. 149–150°, not depressing the m.p. 148° of an authentic synthetic specimen of 3:5-dimethoxyphthalic anhydride.

The acid (21 mg.) was treated with an excess of an ethereal solution of diazomethane. The acid gradually dissolved and N_2 was evolved. After removal of the solvent the colourless alkali-insoluble product (22 mg.) was purified by crystallization from aqueous ethanol giving dimethyl 3:5-dimethoxyphthalate as fine colourless needles, m.p. 92°, not depressed on admixture with authentic synthetic dimethyl 3:5-dimethoxyphthalate, m.p. 91–92°. (Found: C, 56·9; H, 5·6; OCH_3, 48·4. Calc. for $C_{12}H_{14}O_6$: C, 56·7; H, 5·6; $4OCH_3$, 48·8%.)

General properties of oxidation product II. Identification as 2-carboxy-3:5-dimethoxyphenylglyoxylic acid (*VI*). Oxidation product II crystallizes from water as colourless needles, m.p. 173–174° (decomp.). On further heating the melt effervesces and changes to a deep-red liquid which partially resets at about 240° and does not remelt on raising the temperature to 260°. When heated to 100° in a high vacuum 1 mol. water of crystallization is evolved, the crystals become opaque but no change in melting point results. (Found: loss in weight at 100°, 5·7. $C_{11}H_{12}O_8.H_2O$ requires 6·2%. On above dried material: C, 48·6; H, 4·4; OCH_3, 23·7, 23·7; equiv. by titration, 130. $C_{11}H_{12}O_8$ requires C, 48·5; H, 4·4; $2OCH_3$, 22·8%; equiv. titrating as a dibasic acid, 136.) The acid is soluble in $NaHCO_3$ and also in dilute aqueous NaOH to give a pale-yellow solution from which it crystallizes unchanged on acidification. No precipitate is formed on the addition of Brady's reagent to an aqueous solution of the acid. It dissolves in cold conc. H_2SO_4 to a pale-yellow solution which on heating becomes an intense wine-red colour. On shaking the solid acid with a mixture of conc. H_2SO_4 and benzene containing a trace of thiophene the acid layer develops a yellow colour which changes through orange to a strong reddish brown in 15 min. and during 2 days to a deep-green colour. No colour develops in the benzene layer. The corresponding colour sequence described for 2-carboxy-4:5-dimethoxyphenylglyoxylic acid by Kuroda & Perkin (1923) is very similar except that the benzene layer developed a green colour.

The acid was identified as *2-carboxy-3:5-dimethoxyphenylglyoxylic acid* as follows. (*a*) A solution of the hydrated acid (90 mg.), neutralized with 0·1 N-NaOH to phenolphthalein, was cooled in ice-water, and ice-cold 10 vol. H_2O_2 (0·5 ml.) was added. The oxidation solution was held at 0° for 0·5 hr., and then at room temperature for 3 hr. The solution (pH 6·9) was acidified with N-HCl (0·5 ml.) when almost colourless, rectangular microplates separated. The crystals (31 mg.) were filtered, washed with water and dried. The filtrate and washings were exhaustively extracted with ether yielding 44 mg. of a mixture of colourless needles and gum. The first crop of crystals (31 mg.) on heating melted at 182–183° with effervescence and on cooling reset to a mass of colourless needles which remelted at 147°. A mixed melt of the crystals with authentic 3:5-dimethoxyphthalic acid also remelted at 147°. The remainder of this crop was methylated with diazomethane as described above. The neutral methylation product, crystallized from *o*-xylene, melted at 93°, undepressed on admixture with authentic synthetic dimethyl 3:5-dimethoxyphthalate, m.p. 92°. (*b*) A mixture of anhydrous oxidation product II (0·1092 g.), red P (0·25 g.) and constant b.p. HI (4 ml.) was refluxed at 140° in a stream of O_2- and CO_2-free N_2. The effluent gases were passed through a trap containing an aqueous suspension of red P and were then passed through bubblers containing 0·2 N-$Ba(OH)_2$. CO_2 equivalent to 99·6% of 1 mol. prop. was evolved in 3 hr. In order to ensure complete reduction of the product, the mixture was refluxed for a total of 8 hr. with little further evolution of CO_2. The hot solution was then filtered through a sintered-glass funnel and the residue was washed with hot water. The cooled filtrate and washings were extracted with ether (4 × 1 vol.). The combined ether extracts were shaken with water (5 ml.) to remove HI and then with conc. aqueous $Na_2S_2O_3$ (5 ml.) to remove I_2. On removal of the solvent there remained an almost colourless gum (25 mg.) which set to a mass of radiating needles on the addition of a small volume of dry ether. The ether-extracted solution was re-extracted with ethyl acetate (4 × 1 vol.) giving a further crop of gummy needles (41 mg.). A suitable solvent could not be found from which to crystallize the small quantity of product available. It was purified by repeated sublimation in a high vacuum at 120°. 3:5-Dihydroxyphenylacetic acid (VII) was thus obtained as fine colourless needles, m.p. 130–131° not depressing the m.p. of an authentic synthetic specimen, m.p. 128–130°. An aqueous solution gave a moderate violet ferric reaction.

The above 3:5-dihydroxyphenylacetic acid (0·10 g.; m.p. 130–131°) was treated in the usual way with an ethereal solution of diazomethane; the product was crystallized thrice from benzene giving methyl 3:5-dihydroxyphenylacetate as colourless rectangular plates, m.p. 112° not

depressed on admixture with an authentic synthetic specimen of the same m.p. The ester is soluble in cold water, and its aqueous solution gives a stable bluish purple ferric reaction which differs slightly from that given by the free acid. The ester gives a strong brownish red colour in a hydroxamic acid test whereas the parent free acid gave none.

Synthesis of 3:5-dihydroxyphenylacetic acid and its methyl ester. Synthetic triethyl orcinoltricarboxylate (Jerdan, 1899) (0·2368 g.; m.p. 96–98°) was refluxed with red P (0·25 g.) and constant b.p. HI (4 ml.). The CO_2 evolved was estimated as described under section (*b*) p. 430. CO_2 (100·4% of 2 mol. equiv.) was evolved in 0·75 hr. but the heating was continued for a total of 1·5 hr. The reaction solution was filtered and extracted first with ether and then with ethyl acetate as described in section (*b*). The ether extract (77 mg.) consisted of colourless needles of 3:5-dihydroxyphenylacetic acid, m.p. 127–128°, raised to 128–130° by sublimation in a high vacuum at 120° and not depressed on admixture with the corresponding product, m.p. 130–131°, from oxidation product II. The ethyl acetate extract was a partly crystalline gum (40 mg.). von Pechmann & Wolman (1898) recorded that 3:5-dihydroxyphenylacetic acid, when isolated from aqueous solution, melts at 54° but that drying raises the m.p. to 127–128°. The crystalline form and the general properties, including the violet ferric reaction, of the synthetic acid were identical with those of the acid obtained from oxidation product II.

Synthetic 3:5-dihydroxyphenylacetic acid (0·26 g.), dissolved in ethanol (1 ml.), was treated with a 10% excess (for 1 mol.) of an ethereal solution of diazomethane standardized against benzoic acid. On removal of the solvent after 30 min. the yellow oily residue crystallized and was sublimed in a high vacuum at 100–105°. The colourless sublimate of *methyl 3:5-dihydroxyphenylacetate* (0·20 g.), rosettes of needles, melted at 112–113° not depressed on admixture with the methyl ester, m.p. 112°, described above. (Found: C, 59·1; H, 5·5; OCH_3, 16·9. $C_9H_{10}O_4$ requires C, 59·3; H. 5·5; 1 OCH_3, 17·0%.)

Zinc-dust distillation of alternariol methyl ether. Isolation of fluorene

An intimate mixture of alternariol methyl ether (1 g.) and electrolytic Zn dust (20 g.) was subjected to distillation in a combustion tube at a dull red heat in a slow current of H_2, the volatile degradation products passing through an adjacent column of heated electrolytic Zn dust (20 g.). An orange-red oil condensed in the cooler part of the tube and deposited a quantity of colourless needles. The mixture was collected in ether and shaken with aqueous NaOH, acidification of which failed to reveal any phenolic component. On removal of the ether there remained an orange-red crystal-containing oil (0·12 g.) with a cresol-like smell, which was distilled in steam. A red gum (0·01 g.) remained and a mixture of colourless solid and oil separated from the steam distillate. Extraction with ether and removal of the solvent yielded a mixture (0·11 g.) of colourless oil and needles. It was dissolved in a small volume of ethanol, the solution showing a light blue-purple fluorescence in daylight; on cooling, short needles (40 mg.), m.p. 99°, separated. Further crystallization from ethanol yielded colourless needles (10 mg.), m.p. 106–107° raised to 107–108° by sublimation in a high vacuum at 50–55°. (Found: C, 93·8, 93·5; H, 6·3, 6·0. Calc. for $C_{13}H_{10}$: C, 94·0; H, 6·0%.) A mixture of the crystals, m.p. 107–108°, with authentic fluorene, m.p. 113–114°, melted at 110–111°. Also the two specimens had the following properties in common. Both specimens possessed a slight cresol-like smell; ethanolic solutions showed the same weak blue-purple fluorescence in daylight; on heating with conc. H_2SO_4 both gave an intensely blue solution though the Zn-dust distillation product had a somewhat greenish tint probably due to traces of the accompanying colourless oil which itself gave a strong yellow-brown colour on heating with conc. H_2SO_4; both rapidly sublimed in a high vacuum at 55°, the sublimates having identical crystalline forms (spiky needles).

We are indebted to Mr R. Bones, Department of Chemistry, University College, London, for conclusive proof that the zinc-dust distillation product is identical with synthetic fluorene. Mr Bones prepared comparative X-ray powder photographs of the Zn-dust product and of synthetic fluorene using sectors to screen off half the film at a time. The two specimens gave identical diffraction lines, the actual values agreeing with those previously quoted for fluorene by Hofer & Peebles (1951), who claim that a characteristic powder photograph is given by fluorene even when not completely pure.

Degree of unsaturation

Catalytic hydrogenation. A solution of alternariol methyl ether (0·544 g.) in dioxan (35 ml.) was shaken for 1·5 hr. in an atmosphere of H_2 at 22° and 746 mm. with a palladium-norite catalyst. No significant absorption of H_2 was observed and unchanged alternariol methyl ether (0·52 g.) was recovered.

The ester $C_{19}H_{22}O_6$ was shaken with H_2 and a Pd-norite catalyst in methanol, and in a separate experiment with Adams's PtO_2 catalyst. There was no significant uptake of H_2 and the starting material was recovered unchanged in each case.

Ozonolysis of the ester $C_{19}H_{22}O_6$. Ozonized O_2 was passed for 2 hr. through a solution of the ester $C_{19}H_{22}O_6$ (0·29 g.) in $CHCl_3$ (10 ml.) at room temperature. No CO_2 was evolved. The $CHCl_3$ solution, which had developed a pale-yellow colour, was shaken with an equal volume of water at 60°. The $CHCl_3$ layer was shown to contain no $NaHCO_3$-extractable material. On removal of the solvent there remained a colourless, mobile gum (0·26 g.) which solidified on trituration with methanol, and, on crystallization from the same solvent, yielded unchanged starting material (0·15 g.), m.p. 124°.

A portion of the aqueous layer gave no precipitate with Brady's reagent and evaporation of the remainder to dryness gave no residue.

KOH *fusion of alternariol methyl ether. Isolation of 2′:3:4′:5-tetrahydroxy-6′-methyldiphenyl*

Alternariol methyl ether (2 g.), KOH (10 g.) and water (4 ml.) were heated slowly in a nickel crucible to 180° with stirring. A brown frothing liquid was formed. KOH (10 g.) was added, the temperature was raised to 300° in 10–15 min. when the melt had become quiescent. The dark-brown mixture was cooled, dissolved in water (70 ml.) and slowly acidified with conc. HCl (25 ml.). The cooled acid liquid was extracted with ether (5 × 1 vol.). Removal of the solvent yielded a brown gum (1·50 g.) which slowly crystallized in almost colourless needles. The *product* was purified by crystallization from water and sublimation in a high

vacuum at 200° and was obtained as colourless rectangular platelets, m.p. 247–250°. (Found: C, 66·7; H, 5·4; OCH_3, nil; *C*-CH_3, 7·3% = 1·12 *C*-CH_3 groups. $C_{13}H_{12}O_4$ requires C, 67·2; H, 5·2; *C*-CH_3, 6·5%.) The product is insoluble in aqueous $NaHCO_3$ but is soluble in aqueous Na_2CO_3 and in NaOH. Its aqueous solution gives a purple-blue ferric reaction, but an ethanolic solution gives no colour with ethanolic $FeCl_3$. With conc. H_2SO_4 it gives only a pale-yellow solution with a green fluorescence which is only evident in ultraviolet light. On boiling its solution in aqueous NaOH with $CHCl_3$ a cherry-red colour forms in the alkali layer. Unlike alternariol (see p. 429) it shows no antibacterial activity against *Staph. aureus* and *Esch. coli* at the highest concentration tested, i.e. 1:2000.

The product (1 g.) was methylated with dimethyl sulphate (5 ml.), K_2CO_3 (5 g.) and dry acetone (100 ml.). After refluxing under N_2 for 5 hr. the mixture was diluted with an equal volume of water and boiled for 10 min. The resulting brown oil which crystallized on cooling was collected, washed with water and dried, 1·2 g., m.p. 68–70°. The methylated product, *2′:3:4′:5-tetramethoxy-6′-methyldiphenyl*, purified by crystallization from methanol or light petroleum (b.p. 60–80°), formed irregular tablets, m.p. 73–74°, which were insoluble in aqueous 2N-NaOH even on boiling and gave no ferric reaction in ethanol. (Found: C, 70·5; H, 7·1; OCH_3, 42·7, 43·1. $C_{17}H_{20}O_4$ requires C, 70·8; H, 7·0; 4 OCH_3, 43·0%.) Attempts to oxidize the methylated product with $KMnO_4$ led only to uncrystallizable gums.

Action of HI *and red phosphorus*

The method followed was that described on p. 430 for the preparation of 3:5-dihydroxyphenylacetic acid.

(*a*) *On alternariol methyl ether. Isolation of alternariol.* A mixture of alternariol methyl ether (0·1863 g.), red P (0·25 g.) and constant b.p. HI (4 ml.) was refluxed for 3 hr. No CO_2 was evolved. After filtering the cooled reaction mixture, there remained on the filter a mixture of red P and a colourless solid. This was dried, extracted with boiling ethanol and filtered. On removal of the solvent from the filtrate there remained colourless needles (0·179 g.), m.p. 347–349° (decomp.) not depressed on admixture with alternariol.

(*b*) *On the ester* $C_{19}H_{22}O_6$. *Isolation of 2′:3:4′:5-tetrahydroxy-6′-methyldiphenyl.* A mixture of the ester $C_{19}H_{22}O_6$ (see p. 429) (0·1101 g.), red P (0·25 g.) and HI (4 ml.) was used. CO_2 began to be evolved at a bath temperature of 130°, decarboxylation was complete in 0·75 hr. and the reaction was continued for 2 hr. to ensure complete demethylation. CO_2 (0·995 mol. prop.) was evolved. The reaction mixture was filtered. Crystals (65 mg.), m.p. 246–249° (decomp.), separated from the cooled filtrate. They were sublimed in a high vacuum at 200° giving a colourless crystalline sublimate, m.p. 246–249° (decomp.) not depressed on admixture with the KOH fusion product $C_{12}H_{12}O_4$, m.p. 247–250° (decomp.). The two compounds had also identical properties (see p. 431).

Syntheses

2-Bromo-4:6-dimethoxytoluene. $CuSO_4$, $5H_2O$ (1·2 g.), copper bronze (0·4 g.), NaBr (3·1 g.), conc. H_2SO_4 (0·33 ml.) and water (20 ml.) were boiled under reflux for 30 min. Meanwhile, hydrated 2-amino-4:6-dimethoxytoluene sulphate (1·0 g.), prepared according to Curd & Robertson (1933), was stirred in conc. H_2SO_4 (0·25 ml.) and water (10 ml.), cooled in an ice bath and diazotized with $NaNO_2$ (0·35 g.). The Cu_2Br_2 suspension was placed in a steam-distillation apparatus, and when distillation had commenced the diazonium suspension was added and washed in with ice-cold water. Most of the volatile material distilled rapidly, and when a total of 250 ml. had been distilled the distillate was made alkaline with 2N-NaOH (25 ml.) and extracted with ether. The residue after removing the ether (0·22 g.) was used directly for the next stage; it did not solidify on standing at 0° overnight.

2-Bromo-4:6-dimethoxybenzoic acid. The above crude 2-bromo-4:6-dimethoxytoluene (0·22 g.) was refluxed with $KMnO_4$ (0·34 g.) and 0·33N-NaOH (20 ml.) until all the pink colour had gone (2 hr.); at the end of this period much unreacted material remained as oily drops on the surface. The mixture was cooled and treated with sodium bisulphite and 2N-HCl to decolorize, and finally acidified to Congo red. The solution was then extracted with ether (3 × 1 vol.); from the ethereal solution the acidic products were removed with 0·5N-$NaHCO_3$ (2 × 5 ml.). After washing with ether the bicarbonate layer was cleared of ether on the water pump, then acidified, when heavy prisms slowly formed. After standing at 0° overnight these were filtered, washed and dried (23 mg., m.p. 143–148°).

The unoxidized material was re-oxidized with the same amount of $KMnO_4$ and gave a further quantity of acid (33 mg.), m.p. 146–150°. More material (less pure) was obtained on extracting the filtrates with ether. Repeated recrystallization from water gave pure *2-bromo-4:6-dimethoxybenzoic acid* as colourless prisms, m.p. 151–152°. (Found: C, 41·2; H, 3·6; Br, 31·0; OCH_3, 23·5. $C_9H_9O_4Br$ requires C, 41·4; H, 3·5; Br, 30·6; 2 OCH_3, 23·8%.)

4′-Hydroxy-3:5-dimethoxy-6′-methyldibenzo-α-pyrone. 2-Bromo-4:6-dimethoxybenzoic acid (68 mg.), orcinol (68 mg.) and N-NaOH (0·52 ml.) were heated on the steam bath and to the solution was added 0·7% $CuSO_4$, $5H_2O$ (0·52 ml.). A slight flocculent precipitate was produced at once, and during 4 hr. heating on the steam bath no further solid separated. When 2N-HCl (0·52 ml.) was added immediate precipitation of a crystalline solid occurred; after heating for a further 20 min. the mixture was filtered hot. The precipitate was washed with water, then a little 1% $NaHCO_3$ and finally with water again. The crude material (36 mg.) was crystallized from ethanol to give pure *4′-hydroxy-3:5-dimethoxy-6′-methyldibenzo-α-pyrone* as nearly colourless needles, m.p. 293–296°. (Found: C, 67·1; H, 4·9; OCH_3, 21·6. $C_{16}H_{14}O_5$ requires C, 67·1; H, 4·9; 2 OCH_3, 21·7%.) The compound dissolved in conc. H_2SO_4 shows a strong greenish fluorescence in ultraviolet light. It is insoluble in aqueous $NaHCO_3$ and can be crystallized from acetone or acetic acid as well as ethanol. It gives a negative Gibbs test.

3:4′:5-Trimethoxy-6′-methyldibenzo-α-pyrone. 4′-Hydroxy-3:5-dimethoxy-6′-methyldibenzo-α-pyrone (22 mg.) was methylated with dimethyl sulphate (0·1 ml.) and anhydrous K_2CO_3 (0·1 g.) in boiling dry acetone (10 ml.) for 6 hr. The acetone was removed and water (10 ml.) added. Excess dimethyl sulphate was decomposed and the solid was collected and dried. Crystallization from aqueous acetone, then ethanol, yielded colourless needles, m.p. initially 139–140° (incomplete) but resetting on further heating and melting finally quite sharply at 161–162° (a sample of the pure trimethyl ether of alternariol, m.p. 162·5–164° (corr.), was recrystallized from ethanol and showed the same 'double melting point' behaviour). The synthetic material was dissolved in warm acetone, cooled and allowed to evaporate spontaneously at room temperature after seeding

with the natural compound. The colourless needles melted at 162–163° with no softening at 140°. After recrystallization from ethanol (seeding with the higher melting material) a constant m.p. of 162·5–163° (corr.) was obtained. A mixture with alternariol trimethyl ether, m.p. 162·5–164° (corr.), also melted at 162·5–163°; in each case the melt resolidified on removal from the bath and on replacing at 155° partly remelted, then recrystallized slowly; the synthetic compound then remelted at 162–162·5°, the mixture at 162–163°. *3:4′:5-Trimethoxy-6′-methyldibenzo-α-pyrone* therefore exists in two forms, one of m.p. about 140° which is readily transformed above its melting point to that of m.p. 162–163°. (Found: C, 67·8; H, 5·5; OCH_3, 31·0. $C_{17}H_{16}O_5$ requires C, 68·0; H, 5·4; $3OCH_3$, 31·0%.) The substance exhibits a blue-green fluorescence in conc. H_2SO_4 identical with that given by the natural compound.

SUMMARY

1. Two hitherto undescribed mould metabolic products, alternariol and its monomethyl ether, have been isolated from the dried mycelium of laboratory cultures of two different strains of *Alternaria tenuis* grown on Czapek-Dox solution.

2. Alternariol, $C_{14}H_{10}O_5$, forms colourless needles, m.p. 350° (decomp.), which show moderate antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*. Its monomethyl ether, $C_{15}H_{12}O_5$, forms colourless needles, m.p. 267°.

3. A number of derivatives and breakdown products of alternariol and of its methyl ether are described.

4. Alternariol trimethyl ether, colourless needles, m.p. 162·5–163° (corr.), has been synthesized by condensing orcinol and 2-bromo-4:6-dimethoxybenzoic acid and methylating the condensation product.

5. Alternariol has been shown to be 3:4′:5-trihydroxy-6′-methyldibenzo-α-pyrone.

The naturally occurring monomethyl ether has its methoxyl group at position 5 or 4′.

6. Alternariol and its methyl ether are the first recorded substituted dibenzo-α-pyrones of fungal origin.

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Preparation of ^{32}P-labelled Adenosine Triphosphate

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In experiments on the metabolism of adenosine triphosphate (ATP) it became desirable to use the ^{32}P-labelled compound. ATP labelled with ^{32}P has been isolated from the rabbit after injection of radioactive phosphorus by Dounce, Rothstein, Beyer, Meier & Freer (1948), but the specific activity of the product was too low to be of much further experimental use. Therefore a laboratory method of preparing this substance quickly in small quantities of high specific activity was elaborated. It is based on the observation that ATP readily exchanges the two terminal phosphate groups only when incubated with inorganic phosphate in respiring tissue suspensions (Krebs, Ruffo, Johnson, Eggleston & Hems, 1953). In the presence of $^{32}PO_4$ such suspensions convert added ATP in 0·0025 M solution, within about 20 min. incubation, into ATP equally labelled in the two terminal phosphate groups. The active material can be recovered as barium salt and purified by paper chromatography.

The three phosphorus atoms of adenosine triphosphate are distinguished as α, β and γ, thus

adenosine—P^{α}—O—P^{β}—O—P^{γ}.

PROCEDURE

Substrate solution. This is made from 2·5 ml. of a 0·02 M solution of the sodium salt of ATP; 2·5 ml. of 0·02 M-$MgCl_2$; 1·0 ml. of 0·1 M-potassium phosphate buffer, pH 7·4; 0·5 ml. of 0·5 M-sodium α-ketoglutarate; 'carrier free' $KH_2{}^{32}PO_4$ (about 5 mc), and water to a final volume of 11 ml.

Tissue suspension. A sheep heart collected immediately after the slaughter of the animal is transported in ice-cold 0·9% KCl to the laboratory where 50 g. are immediately blended in a Waring Blendor for 2 min. with 200 ml. 0·9% KCl. Smaller quantities cannot be handled in the standard size Blendor.

Incubation. A 10 ml. sample of a freshly prepared tissue suspension and 11 ml. of substrate solution are measured into a 250 ml. conical flask fitted with two glass tubes serving as gas inlet and outlet, and the mixture is shaken at 20° for 40 min. whilst a rapid stream of O_2 is blown through the flask. At the end of the incubation time 2·5 ml. of 30% (w/v) trichloroacetic acid is added and the mixture is chilled in ice. After standing for about 10 min. at 0° the precipitated protein is removed by centrifugation (10 min. at 2000 *g*) in a refrigerated centrifuge at −5°. The supernatant is poured into a 50 ml. conical flask and stored at 0° and the precipitated protein is extracted on the centrifuge with about 5 ml. of water. The combined supernatants are brought to pH 6·8 by the cautious addition of N-NaOH, the pH being followed with a glass electrode. Excess M-barium acetate (about 1 ml.) is added to the solution and the material is stored in a deep-freeze overnight. The purification of the Ba precipitate closely follows the procedure described by LePage (1949), the ATP being once precipitated as the Hg salt by the addition of Lohmann's reagent (100 g. Hg $(NO_3)_2$, $8H_2O$ + 25 ml. conc. HNO_3 (70% (w/w)) + 25 ml. water; the HNO_3 is added to the salt before the water) and once as the Ba salt. The latter is converted to the Na salt by solution in N-HCl and double decomposition with the calculated amount of Na_2SO_4 (0·24 ml. of 0·5 M). The resulting solution (approx. 5 ml.) is placed on a line 10 cm. long at the end of a chromatogram paper washed with ethylenediaminetetraacetic acid as described by Eggleston & Hems (1952). This is most effectively done with a Pasteur pipette drawn down to a capillary fine enough to prevent any escape of liquid unless the pipette is actually in contact with the filter paper. Cold air from a hair-dryer is blown over the surface of the filter paper to facilitate drying. The chromatogram is then developed for 1 hr. by upward paper chromatography using the ethyl ether:formic acid solvent described by Bartley (1953).

Table 1. *Distribution of phosphate and of radioactivity among ATP, ADP and orthophosphate in the solution of* ^{32}P*-labelled ATP*

(Duplicate 5 μl. samples of the solution were chromatographed and the paper areas corresponding to ATP, ADP and orthophosphate were wet-ashed and analysed for phosphorus by the method of Berenblum & Chain (1938), and for radioactivity.)

Substance	Activity in 5 μl. sample (counts/min.)	Phosphorus content (μg.)	Specific activity (counts/μg. P/min.)	Calculated exchangeable phosphorus (μg.)	Calculated specific activity of exchangeable P (counts/μg. P/min.)	Radioactivity in 1·5 ml. of ATP (counts/min.)
ATP	421 000	2·02	208 200	1·34	313 000	126 300 000
ADP	70 900	0·33	214 000	0·16	430 000	21 270 000
Orthophosphate	81 000	0·18	450 000	0·18	450 000	24 300 000

At the end of this time the paper is dried and the band of ATP is located by viewing the paper in ultraviolet light. The strip of paper containing the ATP is cut free from the rest of the paper leaving a narrow border about 2 mm. wide on each side of the band. The remaining portion of the paper is sprayed with the molybdate spray of Hanes & Isherwood (1949) to locate and confirm the separation of the inorganic P from the ATP. The paper strip bearing the ATP is cut so that one end tapers to a point and the ATP is eluted from the paper by downward chromatography with water as a solvent. Progress of the elution is followed by viewing in the ultraviolet light at intervals. Virtually the whole of the ATP is eluted in a volume of 1–2 ml., the radioactivity left on the paper being negligible. The solution is neutralized with 0·1 M-$NaHCO_3$ and is stored in the deep-freeze until required. The solution, about 1·5 ml., is approx. 0·004 M with respect to ATP and contains about 0·2 mc of activity: it is diluted with non-isotopic ATP for use. An analysis of the phosphate compounds in solution by a modification of the paper-chromatographic method of Eggleston & Hems (1952) and the distribution of radioactivity in the fractions is shown in Table 1. ATP, ADP and orthophosphate which had arisen by hydrolysis subsequent to the chromatographic separation, were the only phosphate-containing compounds detected, the phosphorus being distributed as follows: ATP 80, ADP 13, orthophosphate 7%. Of the total radioactivity, ATP contained 77, ADP 11, and orthophosphate 12%.

COMMENTS

(1) The analysis of the ATP used as the starting material gave the following distribution of phosphorus: ATP 68·9, ADP 20·4, pyrophosphate 4·2, orthophosphate 1·5%. The end product was considerably purer; it was free from pyrophosphate. Although it contains only 80% ATP it is purer than the best commercially available preparations tested by us, including samples marketed as 'ATP chromatographically pure'. Further purification by paper chromatography is not difficult, but is usually unnecessary in experiments on tissues or enzyme preparations.

(2) Sheep heart rather than liver or kidney was chosen as tissue because it contains less organic phosphates and other interfering substances such as flavins.

(3) To obtain good yields no large excess of the Lohmann reagent should be used and storage of the mercury precipitate should be avoided.

(4) The chromatographic separation of ATP can be carried out directly on the suspensions acidified with HCl, omitting deproteinization and the initial Ba and Hg treatment. The product obtained, however, is contaminated by salts, and probably by added substrate. This may be irrelevant in some experiments.

(5) The procedure described here may be used for the preparation of other labelled phosphate esters whose phosphate groups exchange with ATP.

(6) Solutions should be kept cold throughout the preparation (approx. 0°) and the pH carefully controlled, especially before the addition of barium acetate.

(7) The highest activity of ATP prepared enzymically depends on the resistance of the enzyme system to the radiation from the isotope. The dose emitted by 10 mc of ^{32}P is approximately 885 R/hr. and as estimated by Dale, Gray & Meridith (1949) a dose of 986 R will inactivate a low concentration of carboxypeptidase. By analogy it may be expected that much higher activity than that used above would damage the phosphorylating systems.

SUMMARY

1. A description is given of a simple laboratory method of preparing ^{32}P-labelled adenosine triphosphate ([β:γ-$^{32}P_2$] ATP). For nomenclature see introductory paragraph of this paper. It rests on the rapid exchange between inorganic phosphate and the β and γ phosphate of adenosine triphosphate by respiring heart-muscle preparations. Non-isotopic adenosine triphosphate and $KH_2{}^{32}PO_4$ are incubated with a heart-muscle suspension. The labelled adenosine triphosphate is successively precipitated as the barium and mercury salt and finally purified by paper chromatography.

2. The product (3·5 mg. adenosine triphosphate) contained between 0·1 and 0·2 mc radioactivity. Of the total activity 77% was in adenosine triphosphate, 11% in adenosine diphosphate and 12% in orthophosphate; of the total phosphorus 80% was in adenosine triphosphate, 13% in adenosine diphosphate and 7% in orthophosphate.

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Studies on Sulphatases

5. THE DETERMINATION OF INORGANIC SULPHATE IN THE STUDY OF SULPHATASES

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The various enzymes which are collectively termed sulphatases are able to hydrolyse certain organic sulphate esters. Four different sulphatases are known: arylsulphatase (Derrien, 1911), glucosulphatase (Soda, 1936), chondrosulphatase (Neuberg & Hofmann, 1931) and myrosulphatase (Neuberg & Wagner, 1927). These enzymes have been reviewed by Fromageot (1950).

Sulphatase studies have been limited by the difficulty of estimating the end products of hydrolysis. Arylsulphatase can be studied by estimating the phenolic end product of hydrolysis and many procedures are available (cf. Dodgson & Spencer, 1953*a*). However, it is more difficult to estimate the organic end products produced by other sulphatases and in the case of certain natural substrates the chemical structure of the organic end product is in doubt. Soda (1936) studied glucosulphatase by determining glucose liberated from glucose 6-sulphate, but this method depended on yeast fermenting small amounts of glucose in the presence of large amounts of glucose 6-sulphate and was complex and time consuming.

Attempts were made to determine liberated sulphuric acid by gravimetric (Soda & Hattori, 1931), nephelometric (Soda, 1936) and manometric (Lindahl, 1935) methods, and by potentiometric titration (Soda, 1936), but the methods were insensitive and necessitated incubation periods of several days. Under such conditions the validity of quantitative enzyme experiment is questionable; furthermore, the suitability of these methods was not fully investigated. Greater sensitivity was achieved by Tanaka (1938) who modified the colorimetric benzidine method, but insufficient experimental evidence was given to justify using the method under widely varying conditions. Recently Lowenstein & Young (1952) adapted the manometric method of Lindahl (1935) to the Warburg apparatus, but the method was available over a limited pH range only.

For studying sulphatases it is desirable to have a method of measuring inorganic sulphate which is sensitive enough for 1 or 2 hr. incubations to be used and which permits many determinations to be made simultaneously. Furthermore, it must be capable of use under varying conditions of pH, molarity of buffer and in the presence of various inorganic ions. A sensitive method of determining blood sulphate has recently been described by Nalefski & Takano (1950), but the method depends on measurement of barium sulphate by photoelectric nephelometer. Cope (1931) and Guillaumin (1940) have defined the errors inherent in precipitating barium sulphate in the presence of biological material and for this reason the method was considered unsuitable. Methods involving precipitation of sulphate as benzidine sulphate and estimation of benzidine in one of several ways (British Drug Houses Ltd., 1948) appeared to be most suitable for the present purpose. Many such methods are described in the literature, but of those examined (e.g. Das Gupta, 1938; Eichelberger, Brower & Roma, 1951; Cuthbertson & Tompsett, 1931) none was directly applicable to enzyme studies. The present paper describes a method which has proved satisfactory, within certain limits for the study and sulphatases.

EXPERIMENTAL AND RESULTS

Materials

Benzidine (Hopkin and Williams, 'Pure' or B.D.H., A.R.). This was twice recrystallized from 95% ethanol (charcoal). For determining sulphate in tissue suspensions a 5% (w/v) solution of benzidine in ethanol was used. The solution was stored in a warm room (otherwise the benzidine tended to crystallize) and was stable for 5–6 weeks. When determining sulphate in aqueous solution a 1% (w/v) ethanolic solution of benzidine was used. All ethanol was filtered or redistilled immediately before use. Powdered glass was washed with distilled water, ethanol and ether and then dried.

Centrifuge tubes. For precipitating benzidine sulphate, 15 ml. centrifuge tubes of even taper with a tip of int. diam. 4–5 mm. were used. All glassware was cleaned with nitric acid; sulphuric-acid-containing mixtures were avoided.

Tissue suspensions. These were prepared according to Dodgson, Spencer & Thomas (1953).

Determination of sulphate in aqueous solution

In a 15 ml. centrifuge tube was placed 1 ml. of a solution of K_2SO_4 (containing from 10 to 70 μg./ml. SO_4^{2-}), 1 ml. of 20% (w/v) trichloroacetic acid and 5 ml. of a 1% (w/v)

ethanolic solution of benzidine. Mixing was by means of a jet of air passing through a fine capillary which was subsequently washed with a few drops of ethanol. The tube was stoppered and allowed to stand for at least 2 hr. at 0°. A small pinch (3–5 mg.) of powdered glass was then added and benzidine sulphate separated by centrifuging at 2250 *g* for 15 min. The supernatant was discarded. In the absence of powdered glass, small particles constantly broke away from the precipitate at this stage, but when powdered glass was present the precipitate packed down tightly on top of the glass and no such losses were experienced. The tube was inverted on filter paper and allowed to drain for 5–10 min. The mouth of the tube was wiped with filter paper and the outside washed with acetone. After standing for 1 min. on filter paper the mouth of the tube was washed with ethanol whilst the tube was held in an inverted position. After draining for a further 5 min. on filter paper, the mouth of the tube was again wiped with filter paper and about 10 ml. of ethanol was added, care being taken not to disturb the precipitate otherwise large fragments broke away which could not effectively be washed. After breaking up the precipitate with a glass rod the whole was mixed and the glass rod washed down with a little ethanol. The benzidine sulphate was separated by centrifuging, and after pouring off the supernatant solution the tube and contents were treated exactly as before except that instead of adding 10 ml. ethanol, the benzidine sulphate was dissolved in 3 ml. of N-HCl. After a few minutes 1 ml. of a 0·1% (w/v) solution of $NaNO_2$ was added. After mixing, 5 ml. of a 0·5% (w/v) solution of thymol in 7·5% (w/v) NaOH was added and the tube centrifuged to remove powdered glass. The intensity of the red colour (λ_{max} 500 mμ.) was measured against a reagent blank with the Hilger Spekker absorptiometer using blue glass filters (Chance O.B. 2) and 2·5 mm. cells. The amount of sulphate was read from a calibration curve prepared by diazotizing and coupling known amounts of benzidine in similar volumes. Between the range 10–70 μg. SO_4^{2-} the calibration curve prepared in this way did not differ from that prepared by actual precipitation and determination of known amounts of K_2SO_4. When present in amounts less than 10 μg., sulphate was not precipitated quantitatively.

Variation of experimental conditions

The effects of varying certain experimental conditions was tried before applying the method to enzyme studies.

Table 1. *The effect of the initial concentration of trichloroacetic acid on the recovery of* K_2SO_4 *from aqueous solutions*

Initial trichloroacetic acid concentration (g./100 ml.)	SO_4^{2-} added (μg.)	Recovery (%)
30	21·8	86·7
26	15·6	93·6
25	31·2	97·2
24	15·6	100·6
20	15·6	98·1
15	15·6	101·9

Concentration of trichloroacetic acid. Trichloroacetic acid concentration was varied between 15 and 25% (w/v) without affecting sulphate recoveries. Below 15% high recoveries were obtained in the presence of 0·01 M-chloride and 0·001 M-phosphate. When the concentration of acid was greater than 25% recoveries of sulphate were low (Table 1).

Concentration of benzidine solution. When the concentration of the added trichloroacetic acid was 20%, low sulphate recoveries were obtained with concentrations of benzidine below 0·75% (w/v).

Time of standing. Good recoveries of sulphate were obtained when the time of standing of the solution, after addition of benzidine, varied between 2 and 48 hr. Recoveries were low when solutions were allowed to stand for shorter periods.

Estimation of sulphate in the presence of buffered tissue suspensions

Recovery of sulphate from pure solution in amounts less than 10 μg. was not quantitative. At concentrations suitable for enzyme experiment, the amount of SO_4^{2-} present in tissue suspensions was usually less than 10 μg. and enzyme control determinations were often low. It was therefore necessary to add sulphate to both tests and controls before precipitating with benzidine. Since sulphate inhibits certain sulphatases (Dodgson & Spencer, 1952) the addition of sulphate was made after completing the incubation. It was convenient to add the sulphate in the trichloroacetic acid solutions. In practice a control value of between 15 and 25 μg. SO_4^{2-} was aimed at.

A mixture of 0·6 ml. of acetate-buffered K_2SO_4 solution and 0·6 ml. of similarly buffered tissue suspension, suitably diluted, was placed in a 15 ml. stoppered centrifuge tube and mixed thoroughly. A buffered suspension without K_2SO_4 was used as a control. After incubation 4·8 ml. of ethanol were added to the tube and the contents were mixed. Protein and, in certain cases, polysaccharide material was precipitated and, after standing for a few minutes to allow flocculation, the tube was capped and the precipitate separated by centrifugation. A 5 ml. portion of the supernatant was transferred to a 15 ml. centrifuge tube and 1 ml. of a 25% (w/v) solution of trichloroacetic acid (containing about 15 μg. SO_4^{2-} as K_2SO_4) followed by 1 ml. of a 5% (w/v) solution of benzidine in ethanol was added to the tube. The contents were mixed by a stream of air as previously described. The tube was stoppered, allowed to stand overnight at 0° and the benzidine sulphate separated and determined as before. The amount of sulphate in the test determination was read from the calibration curve and the corresponding control value was then subtracted. Allowance was made for the contraction in volume which occurred when 1·2 ml. of an aqueous solution was mixed with 4·8 ml. ethanol (cf. Dodgson & Spencer, 1953*a*).

Sulphate could be recovered from a wide variety of tissue suspensions after incubating at 37° for 1 hr. (Table 2). Longer incubation periods did not affect the results, but unsatisfactory recoveries were obtained when the concentration of the tissue suspension in the incubation mixture exceeded 3% (w/v).

Effect of varying buffer concentration and pH. Recoveries of sulphate from incubated tissue suspensions in acetate buffers of varying molarity (0·1–0·75 M) and pH (4·2–7·2) were satisfactory (Table 3).

Effect of inorganic ions on sulphate recoveries. K, Na, Mg, CN, Cl and F at concentrations of 0·01 M, and Fe, Ca and PO_4 at concentrations of 0·001 M did not interfere with the recovery of sulphate from acetate-buffered suspensions

Table 2. *The recovery of* K_2SO_4 *from various tissue suspensions after incubation for* 1 *hr. at* 37·5°

Organism	Organ	Final concn. of suspension (g./100 ml.)	SO_4^{2-} added (μg.)	Recovery (%)
Rat	Liver	2·0	15·8	104·3
	Kidney	2·0	15·8	99·4
	Brain	2·0	15·8	99·4
	Intestine	2·0	15·8	103·8
	Spleen	2·0	15·8	96·8
	Lung	2·0	15·8	101·9
	Pancreas	2·0	15·8	104·4
	Ovary	2·0	15·8	100·0
	Testis	2·0	15·8	101·9
	Heart	2·0	15·8	104·4
Cat	Liver	1·0	35·9	103·3
	Kidney	1·8	31·2	96·8
Littorina littorea	Visceral hump	3·0	23·7	99·6
Patella vulgata	Visceral hump	3·0	23·7	101·7
	Muscular foot	2·0	18·9	100·4
Buccinum undatum	Digestive gland	2·0	23·7	100·0
Cardium edule	Visceral hump	2·0	23·7	101·3
Helix aspersia	Visceral hump	2·0	19·0	98·5
Aspergillus oryzae (Parke-Davis Co. Taka diastase diluted with lactose)		1·5	37·0	101·2

Table 3. *Recoveries of* K_2SO_4 *from rat-tissue suspensions after incubation for* 1 *hr. at* 37·5° *in acetate buffers of varying molarity and* pH

Tissue	Molarity of buffer	pH	SO_4^{2-} added (μg.)	Recovery (%)
Liver	0·10	5·50	19·0	101·6
	0·10	5·20	14·3	101·3
Spleen	0·50	4·20	15·8	100·0
Kidney	0·50	5·20	19·0	103·1
	0·50	5·53	15·8	99·4
Liver	0·50	6·44	15·8	98·1
	0·50	7·20	16·8	98·2
Kidney	0·75	6·44	15·8	98·9

Table 4. *Recoveries of* K_2SO_4 *from acetate-buffered rat-liver suspensions in the presence of various inorganic ions after incubation for* 1 *hr. at* 37·5°

Molarity of buffer	pH	Compound added	Molarity	SO_4^{2-} added (μg.)	Recovery (%)
0·1	5·5	KCN	0·010	19·0	95·3
0·1	5·5	NaF	0·010	19·0	101·6
0·1	5·5	$FeCl_3$	0·010	19·0	55·2
0·1	5·5	$Fe(CH_3COO)_3$	0·010	19·0	55·8
0·1	5·5	$MgCl_2$	0·010	19·0	95·8
0·1	5·5	$CaCl_2$	0·010	19·0	33·2
0·5	5·8	KH_2PO_4	0·010	19·0	*
0·5	5·8	KH_2PO_4	0·001	37·0	95·5
0·5	5·8	$FeCl_3$	0·001	24·7	95·2
0·5	5·8	$CaCl_2$	0·001	24·7	103·7
0·5	5·8	$BaCl_2$	0·001	24·7	0·0

* The final solution was too dense to measure on the Spekker absorptiometer.

(Table 4). However, Fe, Ca and PO_4 in higher concentrations caused considerable interference while Ba, even in traces, rendered the method invalid.

The effect of certain sulphatase substrates. Potassium *p*-acetylphenylsulphate (0·05 M), sodium chondroitinsulphate (1%, w/v), potassium myronate (0·01 M) and potassium glucose 6-sulphate (0·02 M) did not affect recoveries. Higher concentrations of glucose 6-sulphate resulted in high recoveries which were due to low blank values.

Check of the method

Dodgson *et al.* (1953) described a spectrophotometric method for the determination of *p*-hydroxyacetophenone liberated from potassium *p*-acetylphenylsulphate by rat arylsulphatase. The method outlined in the present paper was designed to follow the experimental procedure of the spectrophotometric method, particularly as regards volumes and the use of ethanol as protein precipitant. The two methods were checked against each other after incubating rat-tissue suspensions with potassium *p*-acetylphenylsulphate using the experimental conditions for rat arylsulphatase outlined by Dodgson *et al.* (1953). In each experiment six test and six control determinations were made. After incubating and precipitating protein with ethanol, the inorganic sulphate present in three of the tests and three of the controls was determined by the benzidine method, while the *p*-hydroxyacetophenone present in the remaining tests and controls was measured spectrophotometrically. The results obtained by the two methods were related to each other by calculation and are recorded in Table 5.

Table 5. *Comparison of the* SO_4^{2-} *liberated by rat arylsulphatase (benzidine method) with that calculated from the amount of* p*-hydroxyacetophenone liberated (spectroscopic method) under identical experimental conditions*

(A suitably diluted tissue suspension (0·6 ml.) was incubated with 0·6 ml. of potassium *p*-acetylphenylsulphate (0·007 M) in 0·5 M-acetate buffer (pH 7·2) for 1 hr. at 37·5°.)

	SO_4^{2-} liberated (μg.)	
Source of arylsulphatase	Measured by benzidine method	Calculated from spectroscopically determined *p*-hydroxyacetophenone
Liver	30·7	30·1
	26·6	26·9
	13·3	13·8
	20·2	20·7
Spleen	21·8	23·1
Kidney	33·9	35·9
Lung	15·6	16·3

DISCUSSION

It should be emphasized that in the benzidine methods previously described in the literature which were tried in this laboratory, there is need for extreme care during manipulation. The method described here is no exception. It has been noted consistently that the chief source of error is connected with separating and washing the benzidine sulphate. There is a tendency for the centrifuged precipitate to break away during decantation of the supernatant solution, and although many different types of centrifuge tubes have been tried the only really satisfactory solution to the problem is to use powdered glass as a rough surface on which the precipitate can pack down. Over-generous use of glass must be avoided, however, if the desired effect is to be obtained. More serious is the problem of freeing the precipitated benzidine sulphate from contaminating benzidine. The solution to this would seem to be a second washing with ethanol, but this leads to low results and successive washings of ethanol and acetone are no more satisfactory. However, if the experimental procedure is followed exactly the danger of contamination is reduced to a minimum and once the technique is mastered there is no trouble from this source.

The use of ethanol as a protein precipitant is a departure from the procedure normally followed in the benzidine method. Hitherto, trichloroacetic acid or uranium acetate has been used, but these were found to be generally unsuitable in the present work. Precipitation of protein with uranium acetate necessitates careful adjustment of pH and this is not always easy when solutions of varying pH are involved. Trichloroacetic acid was a satisfactory precipitant with tissue suspensions of rat and dog but, following the removal of protein from certain cat-liver suspensions and preparations from many marine organisms and seaweeds, there was a considerable precipitation of polysaccharide material when ethanolic solutions of benzidine were added. This material later interfered with efficient washing of the benzidine sulphate and agreement between duplicate determinations was poor. However, by altering the sequence of addition of the various reagents and thus using ethanol as protein precipitant, polysaccharide was removed at the same time.

The variations in conditions described here are not exhaustive, and further data become available as the use of the method extends. It is important to test the method (by recovery of added potassium sulphate) whenever new conditions of enzyme experiment are used. However, it is possible to study the various sulphatases under a wide range of experimental conditions, and preliminary work on gluco- and chondro-sulphatases has been reported (Dodgson & Spencer, 1953*b*). No attempts have been made to study the effect on the method of buffers other than acetate since the majority of workers on sulphatases have preferred acetate

buffers which appear to be very suitable for these enzymes.

Determination of sulphate is not adversely affected by most of the common ions used in enzymology. Even when a particular ion interferes the difficulty may often be resolved by using smaller volumes of a more concentrated enzyme solution during the preliminary incubations and then diluting to the correct volumes immediately before addition of ethanol. The necessity for adding inorganic sulphate to both 'test' and 'control' determinations is regrettable but unavoidable. However, provided the sulphate is added after incubation in order to avoid possible enzyme inhibition by sulphate, no serious objection can be raised. The fact that sulphate cannot be recovered when present in amounts less than 10 μg. means that the method cannot be used directly for determining the absolute sulphate content of tissues. Using the volumes suggested earlier and suspensions of the order of 3% (wet, w/v), it was invariably found that the sulphate content of these suspensions was such that less than 10 μg. SO_4^{2-} was actually present in the final solution. It is possible that by adjusting the volumes and using a more concentrated suspension the method might be used to determine tissue sulphate, but since this was not the object of the work the problem was not pursued.

SUMMARY

1. An adaptation of the benzidine method for determining small amounts of enzymically liberated sulphuric acid in the presence of acetate-buffered biological material is described.

2. Using this method potassium sulphate can be recovered quantitatively from tissue suspensions of different organisms under varying conditions of pH and buffer molarity and after incubation for 1 hr. at 37·5°.

3. With the exception of barium, recoveries of sulphate are unaffected by the presence of small amounts of certain inorganic ions.

4. Within certain limits potassium *p*-acetylphenylsulphate, potassium glucose 6-sulphate, potassium myronate and chondroitinsulphuric acid are without effect on the method.

5. The method has been checked under conditions of enzyme experiment by measuring the sulphate liberated from potassium *p*-acetylphenylsulphate by rat arylsulphatase. The values obtained were in good agreement with those obtained by calculation after spectrophotometric measurement of the liberated *p*-hydroxyacetophenone.

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The Assimilation of Amino Acids by Bacteria

17. SYNTHESIS OF GLUTATHIONE BY EXTRACTS OF *ESCHERICHIA COLI*

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Several authors have suggested that glutathione plays a part in the synthesis of proteins. Thus Roberts & Bolton (1952) have shown that when cells of *Escherichia coli* containing ^{35}S-labelled glutathione grow in the presence of unlabelled sulphate, approximately half of the ^{35}S of glutathione is incorporated into the proteins and half is released into the medium. Abelson (1952) reported that a very high proportion of cysteinylglycine residues occur in the proteins of *Esch. coli*. Gale & Folkes (1953*a*) have shown that there is an increase in protein content when washed cell suspensions of *Staphylococcus aureus* are incubated with a complete mixture of amino acids and glucose. If purines and pyrimidines are added to this mixture the nucleic acid content of the cells increases, and a marked acceleration in the rate of protein synthesis also occurs. Bactericidal concentrations of the antibiotics chloramphenicol, aureomycin, and terramycin inhibit protein synthesis in *Staph. aureus* (Gale & Folkes, 1953*b*). Whereas penicillin and bacitracin have no direct effect on the increase in cellular protein obtained when cells are incubated in the presence of a complete mixture of amino acids, the incorporation of labelled glutamic acid into cell protein which occurs when the cells are incubated with glucose and glutamic acid alone is inhibited by these two antibiotics (Gale & Folkes, 1953*c*) and consequently they may be assumed to interfere with some stage in protein metabolism. Hanes, Hird & Isherwood (1950) found that penicillin inhibits transpeptidation reactions involving glutathione in the presence of sheep-kidney suspensions, while Binkley & Olson (1951) showed that the breakdown of glutathione to glutamic acid and cysteinylglycine by this tissue is also inhibited, possibly competitively, by penicillin. It was thought that a study of the synthesis of glutathione by bacterial extracts and the sensitivity of this reaction to antibiotics might throw some light on the possible role of glutathione in protein synthesis. Since there is a structural relationship between glutathione and penicillin it was of particular interest to study the effect of this antibiotic on the synthesis of glutathione. Johnston & Bloch (1951) have obtained a synthesis of glutathione by cell-free extracts of liver. The synthesis of glutathione by cell-free extracts of *Esch. coli* and the action of antibiotics thereon, are the subject of this paper.

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METHODS

Organism. The organism used throughout these studies was *Escherichia coli* N.C.T.C. no. 86 obtained from the National Collection of Type Cultures, London.

Growth conditions. The organism was grown for 15 hr. at 37° in casein digest broth containing 2% (w/v) glucose and aerated with air or O_2. The medium (1·5 l.) was inoculated with 15 ml. of an 8 hr. culture in casein digest broth. Yields of bacteria obtained under these conditions varied from 0·60 to 0·85 mg. dry wt./ml. For some preparations higher yields of bacteria were obtained by growth for 15 hr. at 37° in Roux bottles on the same medium solidified with 3% (w/v) agar.

Growth was determined turbidimetrically on a Hilger Spekker absorptiometer previously calibrated against known dry weights of the organism used.

Preparation of extracts. The organisms were harvested by centrifugation and washed once in cold distilled water containing 0·001 M-cysteine and 0·0001 M-potassium phosphate buffer at pH 7 ('extraction mixture') in a tared tube. Extracts were obtained from the wet cells either by grinding with 2·5 times their weight of powdered alumina in a precooled mortar (McIlwain, 1948) or by crushing with an equal weight of alumina in the Hughes press (Hughes, 1951). In each case the mixture was treated with cold extraction mixture (5 ml./g. wet wt. of bacteria) cooled in ice, and centrifuged at 11 000 *g* on a Servall centrifuge for 5 min. The yellowish, slightly opalescent supernatant fluid was used directly, or after dialysis against cold extraction mixture for 4 hr. with stirring. Such extracts were stored at −18° and normally kept their activity for at least 4 months. Active extracts were also obtained by shaking a thick suspension of the organisms in extraction mixture at 25 kcyc./sec. using a Mullard magnetostrictor oscillator or with glass beads on the Mickle machine (Mickle, 1948). In experiments where the activity of various extracts was compared, a portion of each extract was dried for 3 hr. at 105° and the results expressed per mg. dry weight of the sample.

Incubation mixtures and conditions. The standard medium for incubation was: 1·0 ml. extract; 0·5 ml. 0·5 M-potassium phosphate buffer, pH 7·7; 0·2 ml. 0·16 M-sodium glutamate, pH 7·4; 0·2 ml. 0·16 M-glycine; 0·1 ml. 0·16 M-cysteine, pH 7·4; 0·3 ml. 0·22 M-magnesium hexose diphosphate

(HDP); 0·1 ml. 0·006 M-diphosphopyridine nucleotide (DPN); 0·1 ml. 0·06 M-adenosine triphosphate (ATP); water to 3 ml. The pH of the mixture was between 7 and 7·4.

Incubations were carried out in test tubes (4 × 0·5 in.) in air at 37° for 1 or 1·5 hr. At zero time, a 1·0 ml. sample of the mixture was heated in a test tube in a bath of boiling water for 2 min. with shaking. After cooling, the precipitated protein was removed by centrifugation. The same procedure was adopted for the mixture after incubation. Glutathione was measured in the supernatants and the difference between the final and initial values taken as a measure of the net synthesis of glutathione by the extract.

Estimation of glutathione. Glutathione was measured by the glyoxalase method of Woodward (1935). The possibility was entertained that the relatively high concentrations of phosphate present in the incubation mixture might interfere with the assay of glutathione. It was shown, however, that the addition of similar concentrations of phosphate did not affect the results.

Chromatography. Samples of the incubation mixture were examined by paper chromatography for the presence of glutathione. Samples of the supernatant (0·5 ml.) obtained after deproteinization by heating were treated with twice their volume of warm ethanol containing approximately 0·01 M-*N*-ethylmaleimide which facilitates the migration of glutathione and cysteine on the paper (Hanes *et al.* 1950). Any precipitates formed were removed by centrifugation and the supernatant solutions dried down in a vacuum desiccator. The product was dissolved in 0·1 ml. water and 2–4 μl. spotted on Whatman paper no. 1 or no. 3. Descending chromatograms were developed by a solvent mixture consisting of 80% propanol:20% water (v/v). Papers were dried for 30 min. at 80° and the position of peptides and amino acids determined by spraying with 0·1% ninhydrin in chloroform and heating to 80–100° for 5 min.

RESULTS

Synthesis of glutathione by extracts of Escherichia coli

Early experiments to determine the components required for the synthesis of glutathione by bacterial extracts are shown in Table 1. In most experiments the increase of glutathione during the incubation amounted to 2–5 times that initially present in the extract. In a typical experiment the initial amount was 210 μg. and after 90 min. this had increased to 660 μg. indicating a net synthesis of 450 μg. by 1 ml. of the extract.

Little synthesis occurred in the absence of glycine and/or glutamate, and none after heating the enzyme extract to 100° for 10 min. Bloch, Snoke & Yanari (1952) have shown that ATP is required for incorporation of the amino acids into glutathione by liver extracts. *Esch. coli* contains an active adenosine triphosphatase (ATP-ase) so that added ATP was rapidly destroyed. Good synthesis was obtained, however, if ATP was generated in the incubation mixture by glycolysis of added magnesium HDP in the presence of DPN and a small amount of ATP or adenosine diphosphate (ADP). If neither ATP nor DPN was added to the incubation mixture synthesis was decreased by 50%, and there was little or no synthesis in the absence of both. When HDP was omitted synthesis did not occur even when magnesium ions were added; in the presence of sodium HDP no glutathione was synthesized unless magnesium ions were added. Under the conditions tested, only in isolated cases has it been possible to replace HDP, ATP and DPN by a higher concentration of ATP alone.

Table 1. *Requirements for glutathione synthesis by extracts of* Escherichia coli

(Complete incubation mixture contains: 1·0 ml. extract; 0·5 ml. 0·5 M-potassium phosphate buffer, pH 7·4; 0·2 ml. 0·16 M-sodium glutamate, pH 7·4; 0·2 ml. 0·16 M-glycine; 0·1 ml. 0·16 M-cysteine, pH 7·4; 0·3 ml. 0·22 M-Mg HDP; 0·1 ml. 0·006 M-DPN; 0·1 ml. 0·06 M-ATP and water to 3·0 ml. Incubations were carried out for 90 min. at 37° and results expressed as the amount of glutathione produced in the 3·0 ml. incubation.)

Incubation mixture	Glutathione synthesized (μg.)
Complete system	300
Omit amino acids	6
Omit glycine	30
Omit glutamate	24
Omit DPN	150
Omit ATP	168
Omit ATP and DPN	18
Omit HDP	0
Extract heated to 100°	0

Chromatographic examination of the products after the extract was incubated with added glutathione, showed that no breakdown of glutathione occurred, and consequently this system was particularly suitable for the study of glutathione synthesis.

Effect of time of growth on glutathione content of Escherichia coli

Determinations of the amounts of glutathione in bacteria at different stages in the growth cycle indicated that the cells contain more at the beginning of active growth (5–6 hr.) than when grown for longer periods. Kopper (1951) also found that 3–5 hr. cultures of *Esch. coli* reduced nitrate and methylene blue more readily than did older cells; activities which might be associated with the thiol content of the cells. Initial attempts to obtain an extract capable of synthesizing glutathione were therefore carried out using cultures grown for 5–6 hr. However, it was found that the most active extracts were obtained from cells harvested at the end of their period of active growth (Table 2) and in subsequent experiments 15 hr. cultures were used for the preparation of extracts.

Table 2. *Effect of age of culture on the synthesis of glutathione by extracts of* Escherichia coli

Age (hr.)	Growth (mg. dry wt./ml.)	Activity/mg. dry wt. of extract*
Expt. 1		
3·75	0·24	11·3
6	0·61	13·8
9·5	0·89	18·6
Expt. 2		
4·25	0·44	5·85
8·25	0·72	8·1
13	0·72	11·9

* The activity is expressed as μg. of glutathione synthesized/mg. dry wt. of enzyme extract/hr., under the conditions described in the section on methods.

Rate of synthesis by cell-free extracts

Fig. 1 shows a typical progress curve of the reaction studied at pH 7·3 and 37°. The glutathione synthesis was linear for the first 90 min. and the rate

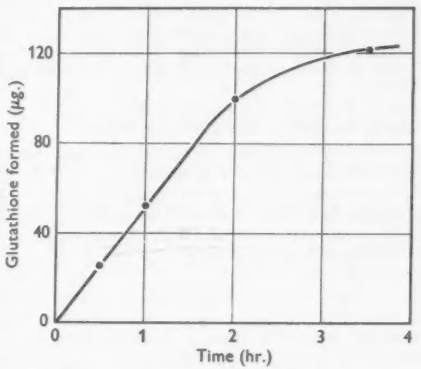

Fig. 1. Progressive synthesis of glutathione from its component amino acids by extracts of *Esch. coli*. Experimental details in text.

then decreased with time, although the reaction continued very slowly up to and over 3 hr. As yet no attempt has been made to determine why the rate of reaction decreases with time. It may be due to oxidation of cysteine, change in pH, lack of high-energy phosphate, or a combination of these factors.

Fig. 2 shows that the amount of glutathione formed is proportional, within experimental error, to the amount of extract present.

Identification of the product

Comparison of chromatograms of the reaction mixture before and after incubation (4 hr.) demonstrates an increase of material running in a similar position to glutathione. Using 75 ml. of extract and corresponding amounts of the other components of the incubation mixture, an attempt was made to obtain larger quantities of the material for chemical identification. The product was precipitated first as the cadmium derivative and then as the copper mercaptide (Waelsch & Rittenberg, 1941). After removal of the copper the material proved a potent activator of glyoxalase and its chromatographic behaviour was identical with glutathione. On hydrolysis the substance gave glutamic acid, glycine, and cysteic acid presumably derived from cysteine.

Effect of antibiotics

The effect of a series of antibiotics on glutathione synthesis by *Esch. coli* extracts was studied. Penicillin, bacitracin, dihydrostreptomycin, streptomycin, polymyxin, neomycin, terramycin, aureomycin and chloramphenicol at concentrations of 100 μg./ml. had no significant effect on the reaction. In view of the fact that penicillin reacts with cysteine (Cavallito, 1946) and might be inactivated, the experiment was repeated using concentrations of penicillin up to 1 mg./ml. No inhibition was observed.

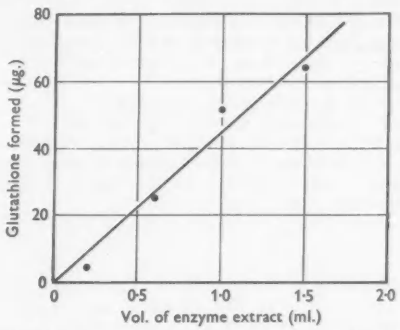

Fig. 2. Effect of enzyme concentration on glutathione synthesis. Incubations carried out for 90 min. at 37°, under conditions described in Methods section.

DISCUSSION

The system extracted from *Esch. coli* which synthesizes glutathione from its constituent amino acids seems to be very similar to that obtained from liver by Bloch *et al.* (1952). No attempt has been made to purify the extract, and it is not possible to say which of the components are essential for the actual building of the peptide and which are concerned with producing the high-energy phosphate bonds necessary to promote the reaction. The fact that HDP, ATP, and DPN cannot be replaced by high concentrations of ATP alone may be due to the very active ATP-ase present in the extracts. Bloch *et al.* (1952) have suggested that in crude extracts

better results may be obtained if the ATP is generated enzymically than if this compound is added to the reaction mixture. Synthesis can occur in the absence of ATP or DPN, but not in the absence of both, and it is possible that DPN may be broken down by the extract to give adenosine phosphates which may be utilized as primers in the HDP fermentation.

Gale & Folkes (1953*b*) have shown that certain antibiotics are potent inhibitors of protein synthesis in staphylococci. None of these antibiotics inhibits the synthesis of glutathione in *Esch. coli*. However, the inhibition studies of Gale were carried out with washed whole-cell suspensions of *Staph. aureus* so that the two systems are not directly comparable. A study of glutathione synthesis in staphylococci might further clarify the problem. The possibility remains that glutathione itself is implicated by transpeptidation reactions in the early stages of protein or peptide formation as suggested by Hanes *et al.* (1950).

SUMMARY

1. Cell-free extracts of *Escherichia coli* synthesize glutathione from glutamic acid, cysteine and glycine in the presence of potassium phosphate, hexose diphosphate, adenosine triphosphate, diphosphopyridine nucleotide and magnesium ions.

2. The results of some kinetic studies on the reaction are given.

3. None of the antibiotics, penicillin, bacitracin, terramycin, aureomycin, chloramphenicol, neomycin, polymyxin, streptomycin, or dihydrostreptomycin, has any effect on the synthesis at concentrations of 100 μg./ml.

I wish to express my thanks to Dr E. F. Gale for advice, criticism and encouragement during the course of this work; to Dr E. Friedmann for supplying *N*-ethylmaleimide; to Dr F. J. R. Hird for guidance with the chromatography; and to Mr E. J. Morgan for assistance in isolating the glutathione. My thanks are due also to the Medical Research Council for the award of a training grant and to Girton College, Cambridge for a scholarship.

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Occurrence of Two Valeric Acids (β-Methylbutyric Acid and α-Methylbutyric Acid) in the Hair Grease of the Dog

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The fatty excretions of the skin of some animals contain not only volatile fatty acids (free and combined) with an even number of carbon atoms, but also volatile fatty acids with an odd number of carbon atoms, in particular valeric acid (Brouwer & Nijkamp, 1952). This acid was found in the 'hair grease' of goats, of sheep, and particularly in that of dogs, where the volatile acid is almost exclusively valeric acid and amounts on an average to 5 % of the lipid fraction. The question as to which of the isomeric valeric acids occurred was left unanswered, although it was pointed out that *iso*valeric acid (β-methylbutyric acid) was not unknown in the animal kingdom. Substantial quantities of it occur in the body fat of dolphins and porpoises (Lovern, 1934). A closer investigation has therefore been made into the nature of the valeric acid in the hair grease of the dog.

EXPERIMENTAL AND RESULTS

Preparation of the volatile fatty acids

Dog hair (15·06 kg.) from animals of various breeds was extracted with peroxide-free ether in five lots to yield 304·9 g. of hair grease. This fat was saponified, again in lots, by a solution of KOH in acid-free ethanol for 15 hr. The removal of ethanol by distillation was difficult and water had to be added several times before the odour of the alcohol was no longer noticeable. With the second and later fractions ethanol was evaporated under vacuum.

The soap was then hydrolysed with hot dilute H_2SO_4 and the volatile fatty acids were steam distilled and absorbed in 0·1 N-NaOH. The solution thus obtained was evaporated down to a viscous mass, from which the fatty acids were extracted by peroxide-free ether after acidification with $KHSO_4$. The extract was dried with Na_2SO_4 and the ether distilled off at temperatures rising to 100°. Titration of the distillate showed that very little volatile fatty acid had distilled over. The residue, mainly consisting of the fatty acids, weighed 11·548 g., i.e. 3·79% of the original hair grease.

Chromatographic tests (Nijkamp, 1951) carried out on the five separate portions, showed that the fatty acids consisted almost entirely of valeric acid, but very small quantities of acetic acid, propionic acid and octanoic (caprylic) acid were also detected. After drying over $CaCl_2$ the equivalent weight was 104·0 (calc. for valeric acid 102·0).

Analysis of the volatile fatty acid

To determine which isomer (or mixture of isomers) was present it was necessary to start from the pure isomers to see how thorough a separation was possible on the silica-gel column (Nijkamp, 1951). In this way it became evident that trimethylacetic acid moved much more quickly down the column than the other three acids and therefore could easily be separated. It was clear, however, that the preparation from dog hair grease did not contain any trimethylacetic acid.

The separation of the other three isomers was usually incomplete, but the α-methyl isomer moved a little more quickly than the β-methyl isomer. The *n*-valeric acid probably moves at an intermediate rate.

Determinations of physical properties are set out in Table 1 which includes also data on three preparations of commercial *iso*valeric acid, which is known to be mainly β-methylbutyric acid, mixed with varying proportions of α-methylbutyric acid. These preparations showed a more or less complete separation on the silica-gel column into two bands the β-isomer greatly predominating over the faster-moving α-isomer.

The table shows that only the boiling points of α- and β-methylbutyric acid and of '*iso*valeric acid' fall within the range of the boiling point of the preparation from dog hair grease. The boiling point of trimethylacetic acid is considerably lower, and that of *n*-valeric acid substantially higher. The boiling points of the α- and β-isomers are not very far apart. On the other hand, the differences between the refractive indices of these two acids are very marked, and from this alone it can be concluded that the dog preparation consists mainly of β-methylbutyric acid.

Chromatography of the valeric acid preparation from dog hair showed that the front of the band on the silica-gel column was rather faint indicating a fraction moving more quickly than the β-isomer. From the boiling point and refractive index it seemed likely that this fraction was the α-isomer. This is in fact the only valeric acid with an asymmetric carbon atom. In most instances the dextrorotatory modification has been found in nature; $[\alpha]_D + 17·85°$ (Schmidt, 1922). The valeric acid preparation from dog hair had α_D +0·738° (homogeneous) in a tube of 1 dm. length. Since the sp.gr. of the preparation proved to be 0·93, the specific rotation was +0·794°. If, therefore, only active α-isomer was present, its amount must have been 4·45%. It is possible that in addition to this racemic α-isomer might also have been present.

Infrared-absorption spectra

Confirmation of the foregoing was obtained by an investigation of the absorption in the infrared kindly carried out in the laboratory of Prof. Ketelaar at Amsterdam at our request by Mr Vedder and Mr Hellingman. The absorptions of the preparations referred to were recorded between the wavelengths 2·5 and 14·5 μ.

The spectra of the isomeric valeric acids showed very marked differences but the shape of the curve for β-methylbutyric acid was essentially identical to that of our preparation. The latter therefore consisted mainly of β-methylbutyric acid. Nevertheless, the two curves showed slight differences, from which it became apparent that, in addition, some α-methylbutyric acid must be present. This was most

Table 1. *Properties of valeric acids*

	Boiling points: Estimated in main fraction (corr.) (°)	Boiling points: Recorded in literature (°)	Refractive index (main fraction) n_D^{20}
Trimethylacetic acid	163·7–163·9	163·7,* 163·8†	—
α-Methylbutyric acid	175·7	177·0,* 173–175†	1·4057
β-Methylbutyric acid	175·6–175·8	176·7,* 175†	1·4038
n-Valeric acid	183·9–185·7	186·4,* 184–185†	1·4093
'*iso*Valeric acid' (1)	175·0–175·3	—	1·4040
'*iso*Valeric acid' (2)	175·6–176·0	—	1·4039
'*iso*Valeric acid' (3)	175·7–176·2	—	1·4037
Volatile fatty acid of dog hair	172·9–176·7	—	1·4038

* Rodd (1952). † Schmidt (1922).

plainly in evidence between 12·6 and 13·4 μ., where the absorption of the α-methylbutyric acid shows a maximum at 12·9 μ.

Curves were also recorded in this region for: *A*, pure β-methylbutyric acid; *B*, β-methylbutyric acid (95%) +α-methylbutyric acid (5%); *C*, β-methylbutyric acid (80%) +α-methylbutyric acid (20%); *D*, volatile fatty acid from dog hair grease (main fraction).

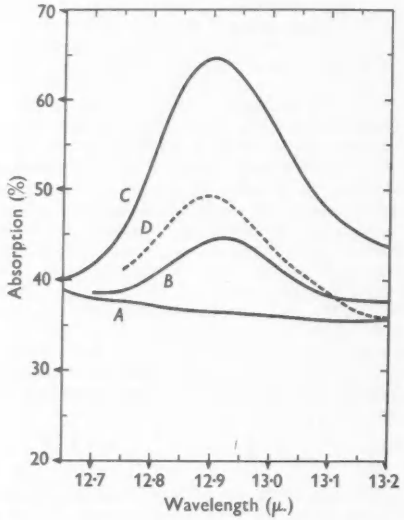

Fig. 1. Absorption of valeric acids in the infrared between 12·7 and 13·2 μ. The curves *A*, *B*, *C* refer to β-methylbutyric acid containing respectively 0, 5 and 20% of α-methylbutyric acid; curve *D* is for volatile fatty acids (main fraction) from dog hair.

No absolute value can be attributed to the ordinates in these curves (Fig. 1), as the dog hair grease fatty acid contained another unknown substance or substances which caused some extra light absorption over the whole range of the curve. Some recalculation of the values recorded was necessary in order to permit comparison with the curves of the other acids. Moreover, the extra absorption of light was stronger on the short-wave side resulting in a slight displacement of the absorption maximum for our preparation.

Nevertheless, it can be seen from the curves that the amount of α-methylbutyric acid in our preparation must have been somewhat more than 5%. This is slightly greater than could be deduced from the optical rotation. The difference is not big enough, however, to justify attributing it with certainty to the racemic α-isomer, though its presence in very small amount cannot be excluded.

Lastly the curves provide no indication whatsoever of the presence of *n*-valeric acid or trimethylacetic acid in the preparation from dog hair grease. If they had been present at all, the quantities must have been extremely small.

DISCUSSION

With reference to the precursors of β-methylbutyric acid or of '*iso*valeric acid' in Delphinidae, Hilditch (1949) states: 'The fact that the carbon skeleton of this acid is the same as that in isoprene may, of course, be purely coincidental, or, on the other hand, it may be an indication that this acid is derived from a precursor belonging to the terpene series.' Boekenoogen (1948) adopted this point of view and sought a relationship with polyterpenoids (such as squalene) which are plentiful in train oils.

Much more plausible is the hypothesis that, at least with dogs, the β-methylbutyric acid is formed from leucine and the α-methylbutyric acid from *iso*-leucine. In both cases the transformation requires deamination, decarboxylation and oxidation, the carbon chain losing one atom. Some support for our hypothesis is given by the results of Neuberg (1911) who was able to prove the formation of α-methylbutyric acid from *iso*leucine by bacilli. Neuberg also states that von Nencki had observed the conversion of leucine into valeric acid in a putrefaction experiment as early as 1876.

Bacterial conversions play only a minor part in the intestine of the dog but this is not a serious objection to the hypothesis, as it is well known that deamination, decarboxylation and oxidation also take place in animal tissues, and therefore the formation of the α- and β-isomers could also be accomplished in this way (Bodansky, 1938).

SUMMARY

1. It has been confirmed that the volatile fatty acids in the skin excretions (hair grease) of dogs consist mainly of valeric acid with very small quantities of acetic acid, propionic acid and octanoic (caprylic) acid. The aggregate amounted to nearly 4% of the hair grease.

2. Most of this valeric acid consists of β-methylbutyric acid. In addition there is present, however, about 5% of (+)α-methylbutyric acid.

3. It is suggested that leucine and *iso*leucine may be the parent materials of these two fatty acids.

We express our gratitude to Messrs Koolmoes at the Hague for their kindness in supplying us with the dog hair required, to Prof. J. A. A. Ketelaar, Mr W. Vedder and Mr Hellingman at Amsterdam for the infrared-absorption curves, to Prof. E. Havinga and his collaborators at Leyden for the preparation of pure α- and β-methylbutyric acid and to Prof. J. P. Wibaut and Dr H. C. Beyerman (Amsterdam) for the use of micropolarimetric apparatus.

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A Comparison of the Effects of Cortisone and Deoxycorticosterone on Various Aspects of Organic and Inorganic Metabolism

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Since Hartman, Spoor & Lewis (1939) first demonstrated that adrenal cortical extracts contained at least two hormones, one of which they termed a 'sodium factor', it has been customary to divide adrenal cortical hormones into two functional groups: 'mineralo-corticoids' of which 11-deoxycorticosterone is considered a typical example, although it appears to have been isolated only once from the adrenal cortex (Reichstein & von Euw, 1938), and 'gluco-corticoids' such as the corticosterone group. It was considered that the deoxycorticosterone type of hormone had no appreciable action on carbohydrate metabolism or on glycogen deposition in the liver (Thorn, 1944; Kendall, 1941; Ingle, 1945; Olson, 1944; Grollman, 1947; Reinecke & Kendall, 1943; Olson, Thayer & Kopp, 1944), and that the gluco-corticoid hormones, e.g. cortisone, had no appreciable effect on mineral metabolism, and would be expected to have little or no influence on the inorganic levels of the blood plasma. Montigel & Verzár (1943) concluded that the difference in action on carbohydrate metabolism of these two groups of hormones is not qualitative, but rather one of velocity, deoxycorticosterone acting more slowly. Wang & Verzár (1949) showed that deoxycorticosterone is capable of restoring to normal the liver and muscle glycogen content of adrenalectomized rats and Sass-Kortsak, Wang & Verzár (1949) found that in long-term experiments the difference in action of deoxycorticosterone and cortisone in this respect is not marked. Similarly, Nissim (1952) has recently demonstrated the glycogenic effect of large doses of deoxycorticosterone acetate in adrenalectomized mice. Recent studies (Roberts & Pitts, 1952; Tait, Simpson & Grundy, 1952) have indicated the efficiency of cortisone in decreasing the renal excretion of sodium in adrenalectomized animals. Much evidence has also appeared from the clinical side (Hartman & Brownell, 1949; Forsham *et al.* 1949; Perera, Pines, Hamilton & Vislocky, 1949; Sprague & Power, 1950; Sprague *et al.* 1950; Fourman *et al.* 1950), that the so-called gluco-corticoid hormones may in fact exhibit considerable effect on sodium retention and potassium excretion.

The present communication gives the results of a study of the comparative effect of the two representative hormones, cortisone and deoxycorticosterone, on the levels of various carbohydrate intermediates in the skeletal muscle of normal and adrenalectomized rats (Conway & Hingerty, 1946), on the sodium and potassium levels of the plasma and muscle, with low- and high-sodium diets, and on growth rate and nitrogen metabolism. Finally, some permeability studies were carried out with the hormones on frog muscle, using ^{24}Na and ^{42}K.

METHODS

Treatment of animals

Young albino rats of the Wistar strain about 6 weeks old and weighing about 80–90 g. were used; for the effects on growth rate and nitrogen metabolism only female rats were used. The rats were maintained for a few weeks before and throughout the experimental period on a diet of the following composition: oatmeal, 44·4; oat flour, 44·4; Bemax, 3·9; baker's yeast, 1·7; cod-liver oil, 5·6%. When the effects with a diet rich in Na were being examined, 12 g. NaCl/kg. was added to the basal diet.

Adrenalectomies were performed under ether-air anaethesia in a single-stage operation, after which the animals were maintained at constant temperature. Cortisone and deoxycorticosterone were used in the form of cortisone acetate prepared by Merck & Co., Inc. and deoxycorticosterone acetate (DOCA) prepared by Organon

Laboratories. The hormones were injected intramuscularly at the same time each day, being administered as a single 2 or 2·5 mg. injection of the acetate, whilst adrenocorticotrophic hormone (ACTH) (Armour Laboratories) was given in two divided doses, one every 12 hr. An amount equivalent to 0·5 i.u. was injected twice daily in one series, and an amount equivalent to 1·0 i.u. was injected twice daily in a second series. Urine was collected at the same time each day from groups of 7 rats, in a metabolism cage, in flasks containing thymol and citric acid as preservatives. The total daily volume was measured and made up to 50 ml., from which samples were taken for the various analyses. In obtaining samples of blood and muscle the rats were stunned and bled directly into paraffined centrifuge tubes following which about 0·25 g. of fat-free muscle was removed into a weighed platinum crucible and dried overnight at 105° to obtain the water content. The muscle was ashed for Na and K determinations. In estimating the phosphate compounds in muscle, about 1 g. of the tissue was removed immediately into ice-cold trichloroacetic acid.

Chemical methods

The Barclay flame photometer with internal lithium standard was used for the Na and K determinations. N and Cl were determined by microdiffusion methods (Conway, 1950) and blood glucose by a modification of the Folin & Malmros (1929) method. The various organic phosphate compounds in the muscle were estimated after fractionation of a trichloroacetic acid extract as previously described (Conway & Hingerty, 1946) with some modifications.

In the tracer experiments, frogs were injected with ^{42}K or ^{24}Na in sufficient radioactive strength to give easily determinable counts from the sartorii excised the following morning. Such sartorii were immersed with gentle shaking at 18° in a relatively large volume of Ringer fluid and the count curves, suitably corrected for decay, compared with similar curves obtained from frogs injected with cortisone in doses of up to 3·75 mg., administered in three separate injections of 1·25 mg. 24, 18 and 6 hr. before removing the muscles.

RESULTS

Effects on the phosphate distribution in muscle of normal and adrenalectomized rats

The following determinations were made: total phosphate (TP), acid-soluble phosphate (ASP), residual phosphate (RP; TP − ASP), phosphocreatine (PC), adenosine triphosphate (ATP), glucose 6-phosphate (G 6-P), glucose 1-phosphate (G 1-P) and fructose 6-phosphate (F 6-P). The experiments were carried out on normal and adrenalectomized rats, untreated and injected with cortisone or deoxycorticosterone.

The average results (each figure being obtained from 7–11 experiments) are shown in Table 1, together with the mean differences and probability values.

It will be seen that treating normal rats with deoxycorticosterone or cortisone has no significant effect on the various substances examined. Adrenalectomy causes a very marked fall in glucose

Table 1. *Changes in phosphorylated compounds in skeletal muscles of normal and adrenalectomized rats*

(Cortisone or deoxycorticosterone given as five daily injections of 2·5 mg. All values are m-moles P/kg. muscle. *P* values given in brackets after mean differences.)

	ASP	RP	PC	ATP	G 6-P	G 1-P	F 6-P
Normal	55·6	25·2	25·2	19·5	4·48	2·61	0·95
Change after adrenalectomy	−0·8 (0·70)	+0·9 (0·17)	+3·4 (0·06)	+1·2 (0·06)	−4·1 (0·01)	+0·27 (0·45)	−0·73 (<0·01)
Change after cortisone injection	−1·4 (0·86)	+0·8 (0·71)	−0·5 (0·57)	−0·7 (0·52)	−0·22 (0·70)	+0·53 (0·14)	−0·11 (0·14)
Change after deoxycorticosterone injection	−2·0 (0·83)	+4·9 (0·74)	+0·6 (0·40)	+0·3 (0·22)	−0·12 (0·56)	+0·20 (0·72)	−0·21 (0·11)
Adrenalectomized	54·8	26·1	28·6	20·7	0·77	2·88	0·22
Change after cortisone injection	−0·2 (0·80)	+1·2 (0·21)	−2·5 (0·28)	−1·2 (0·49)	+2·07 (<0·01)	−0·06 (0·44)	+0·28 (<0·01)
Change after deoxycorticosterone injection	−0·6 (0·64)	+1·5 (0·66)	−1·1 (0·88)	−0·5 (0·64)	+1·42 (0·11)	+0·30 (0·07)	+0·20 (0·01)

6-phosphate (to 16 % of normal level) and in fructose 6-phosphate (to 23 %); an increase in phosphocreatine, which is probably significant, also occurs. Although the phosphocreatine level is increased by only 13 %, this represents an absolute increase of 3·4 m-moles phosphorus/kg. as compared with an absolute fall in glucose 6-phosphate of 4·1 m-moles phosphorus/kg. This is in agreement with previous work (Conway & Hingerty, 1946). In addition there is an increase in the ATP level after adrenalectomy, which is probably significant.

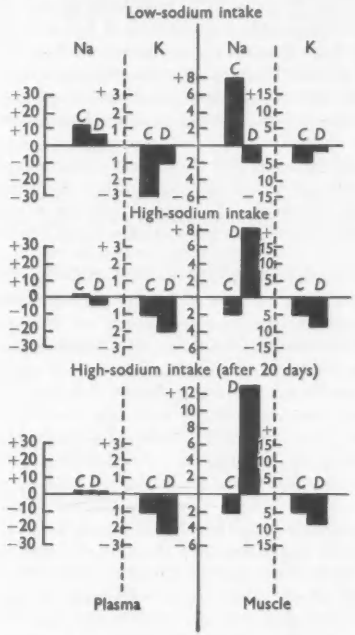

Fig. 1. Effects of cortisone (*C*) and deoxycorticosterone (*D*) injections on the K and Na levels in plasma and muscle of normal rats on low-Na and high-Na intakes. Ordinates: mean increase or decrease in concentration in m-moles/kg., compared with normal.

It therefore appears that two substances of major consequence containing labile energy-rich phosphate groups in muscle (ATP and phosphocreatine) are increased by adrenalectomy, while glucose 6-phosphate, the product of the phosphorylation of glucose by the hexokinase system, is greatly decreased. So also is fructose 6-phosphate.

The question then arises as to how far the injection of cortisone or of deoxycorticosterone replaces the total loss of adrenal hormones in restoring these metabolites to the normal level. Cortisone in the dosage given restores the glucose 6-phosphate and fructose 6-phosphate levels 50 and 38 %, respectively. Deoxycorticosterone, although qualitatively similar in action, is appreciably less effective, restoring glucose 6-phosphate and fructose 6-phosphate only 34 and 27 %, respectively.

Effects on the levels of potassium and sodium, in plasma and muscle, of normal and adrenalectomized rats

The results with high-Na and low-Na diets are shown in Figs. 1 and 2. Fig. 1 illustrates graphically the effects of cortisone and deoxycorticosterone injections (five daily injections of 2·5 mg.) on the Na and K levels of plasma and muscle for the normal rat with low-Na and high-Na intakes. Also shown in Fig. 1, are the effects of 20 daily injections of 2·5 mg. of cortisone and deoxycorticosterone with the high-Na diet. With low-Na intake both cortisone and deoxycorticosterone increase the

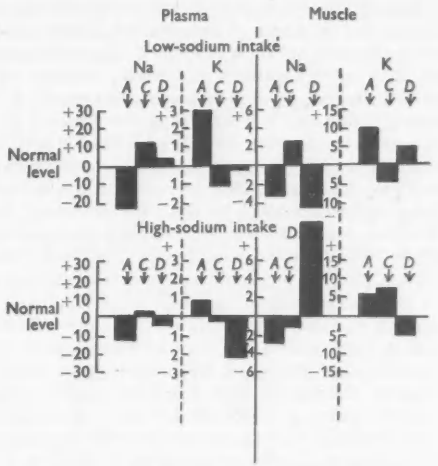

Fig. 2. Effects of adrenalectomy (*A*) and of subsequent cortisone (*C*) and deoxycorticosterone (*D*) injections on K and Na levels in plasma and muscle of adrenalectomized rats on low-Na and high-Na intakes.

plasma Na levels but the relative effect of cortisone is greater. With much Na in the diet the hormones have no appreciable effect on the plasma Na, even after 20 daily injections. With plasma K the effect of each hormone is to lower the level but the relative effects of the hormones when the diet is poor in Na is reversed when the Na content of the diet is high. The effects of the two hormones on muscle Na and muscle K are also conditioned by the Na intake. With a low-Na diet, cortisone markedly increases the level of muscle Na whereas deoxycorticosterone lowers it somewhat and the reverse applies when the diet is rich in Na. Finally with regard to the muscle

K, this is lowered by each hormone, the relative effects again being reversed on changing to the Na-rich diet.

Some effects on the muscle water also occur, but even on expressing the quantities per kg. dry matter the above relations are not significantly affected.

The different effects of cortisone and deoxycorticosterone, depending on the level of Na intake, illustrates very strikingly the intricate nature of the picture presented. In this respect the findings of Selye, Stone, Timiras & Schaffenburg (1949) and of Fourman & Kepler (1949) that the storage of sodium chloride in overdosage of deoxycorticosterone is dependent on conditioning by simultaneous sodium chloride administration are of interest. Similarly, Ransohoff *et al.* (1950) found that many of the metabolic and physiological effects of ACTH may be strikingly influenced by the Na intake.

The effects of cortisone and deoxycorticosterone on Na and K levels of muscle and plasma after adrenalectomy are shown in Fig. 2. The well-known effect of adrenalectomy in lowering plasma and muscle Na while increasing plasma and muscle K is shown, being much greater with the Na-poor diet than with the Na-rich diet. Both cortisone and deoxycorticosterone reverse the post-adrenalectomy changes, the relative effectiveness of the hormones being again dependent on the level of dietary Na. With low-Na intake, cortisone shows a much greater effect than deoxycorticosterone and with the dosage used the levels are markedly over-restored. In these experiments the daily urine volume and urinary excretion of Na, K and Cl were followed over a 5-day period and over a 20-day period, the results being compared with those from control animals. Taking the first few days' results in the present series as least affected by variations in dietary intake, they confirm what might be deduced from the blood picture with regard to the action of the hormones on the retention or release of K.

Where a statistically significant effect has been observed, the changes in levels of K and Na in muscle and plasma have been recorded in Table 2 simply as + and − signs, representing increases or decreases compared with the normal values. Also included are the effects of a prolonged K-free diet and of the return to a high K diet (Conway & Hingerty, 1948).

From this it appears that there exists a constant relationship between the changes in plasma K and muscle Na, any alteration in plasma K level, however brought about, being accompanied by a change in muscle Na in the reverse sense. In the four groups in which there was a considerable fall in the plasma K (over 30 %, indicated by − −) there was a considerable rise (indicated by + +) in the muscle Na. From a consideration of such facts it would appear that the plasma changes which take place in adrenal insufficiency or following hormone administration could of themselves bring about the muscle changes.

It may be noted that the largest changes in muscle Na, such as those occurring after deoxycorticosterone injections into normal rats on a high-Na diet, can occur without any change in the plasma Na.

Effects on muscle-water content

The effects of five daily 2·5 mg. injections of cortisone or deoxycorticosterone on the muscle-water content of normal and adrenalectomized rats on low-Na and high-Na intakes were investigated. The results are shown in Table 3 from which it appears that: (*a*) on the high-Na diet the water content of muscle from normal rats is higher than in muscle from animals on the low-Na diet. (*b*) Adrenalectomy raises the water content of muscle both on low-Na and on high-Na diets, the effect being more marked on the low-Na diet. (*c*) On the low-Na diet both cortisone and deoxycorticosterone are effective in reducing the water content of muscles of adrenalectomized rats, cortisone being more effective in this respect. (*d*) On the high-Na diet cortisone reduces the water content of muscle from adrenalectomized rats but deoxycorticosterone is ineffective.

The greater efficacy of cortisone in reducing the muscle water may be related to the findings of

Table 2. *Sodium and potassium changes in plasma and muscle under various conditions*

(+ and − signs indicate statistically significant changes compared with normal level; + + and − − indicate changes of 30 % or greater; 0 indicates no significant change.)

Diet	Conditions	Plasma		Muscle	
		Na	K	Na	K
Low-Na	Adrenalectomy	−	+	−	+
Low-Na	Adrenalectomy + cortisone	+	−	+	−
High-Na	Adrenalectomy	−	+	−	+
High-Na	Adrenalectomy + deoxycorticosterone	0	− −	+ +	−
Low-Na	Cortisone injections into normal rat	+	− −	+ +	−
High-Na	Deoxycorticosterone injections into normal rat	0	− −	+ +	−
K-free		0	− −	+ +	−
K-free followed by K-rich		0	+	−	+

Table 3. *Changes in water content of rat muscle following adrenalectomy and injections of cortisone or deoxycorticosterone into normal and adrenalectomized rats on low-Na and high-Na diets*

(Five daily 2·5 mg. injections of cortisone or deoxycorticosterone. All values given as g. water/100 g. muscle. *P* values in brackets.)

	Low-Na diet	High-Na diet
Normal	75·87	77·01
Change after adrenalectomy	+1·81 (0·01)	+0·58 (0·20)
Change after cortisone injections	+0·90 (0·67)	−1·33 (0·12)
Change after deoxycorticosterone injections	+0·27 (0·71)	−0·17 (0·85)
Adrenalectomized	77·68	77·59
Change after cortisone injections	−2·15 (<0·01)	−2·34 (<0·01)
Change after deoxycorticosterone injections	−1·54 (<0·01)	+0·35 (0·53)

Eversole, Gaunt & Kendall (1942) who found cortisone to be at least 3 times as effective as deoxycorticosterone in protecting adrenalectomized rats against water intoxication.

Effects of cortisone, deoxycorticosterone and ACTH injections on the growth rates of young rats

Fig. 3 summarizes the effects of the injections on body weight for both normal and adrenalectomized rats, over a 5-day period, each point being the mean

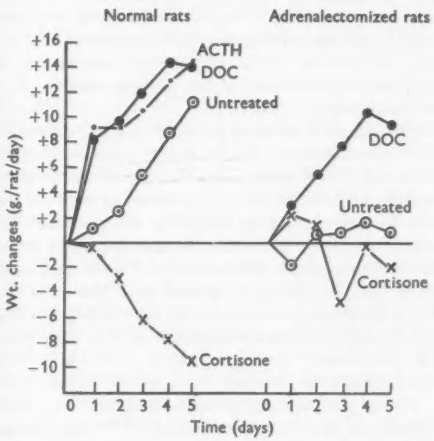

Fig. 3. Effects of cortisone and deoxycorticosterone injections on growth rates of normal and adrenalectomized young rats and of ACTH on growth rate of normal young rats.

for seven rats. It will be seen that in the normal rats both deoxycorticosterone and ACTH increase the rate of growth but cortisone markedly decreases it. After adrenalectomy the cortisone-treated rats tend to a small decrease in weight (the untreated animals show practically no change) but the decrease is much less than in the normal rats. The injection of deoxycorticosterone into the adrenalectomized rats enables them to maintain a practically normal weight increase.

Fig. 4 shows the effects on weight over a much longer period, each point being the average for seven rats. The very pronounced effect of cortisone in decreasing the weight is manifest, the result being strikingly different from that of deoxycorticosterone.

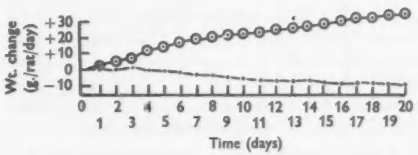

Fig. 4. Effects of cortisone (·) and deoxycorticosterone (⊙) injections on weight changes in normal young rats; each animal received 2 mg. of the hormone/day for 20 days. Each point represents seven rats.

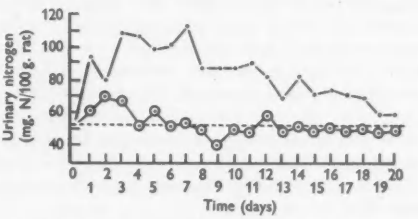

Fig. 5. Effects of cortisone (·) and deoxycorticosterone (⊙) injections on urinary N excretion in normal young rats; each animal received 2 mg. of the hormone/day for 20 days. Each point represents seven rats.

Effects on nitrogen excretion

The effects of cortisone and deoxycorticosterone injections on the urinary nitrogen excretion were studied on the group of rats used in Fig. 4, the results being expressed as mg. nitrogen/100 g. body wt./day. The results, which are given in Fig. 5, where each point is likewise the average for seven rats, show the great effect of cortisone in increasing the nitrogen excretion.

Effects of cortisone on the permeability of frog sartorius muscle to sodium and potassium ions

Preliminary studies on the effects of cortisone on the permeability of the frog sartorius were carried out, the relative entry and exit rates of Na and K into and out of the fibres being measured for normal and cortisone-injected frogs. The problem of measuring the relative entry and exit rates was simplified by the use of the radioactive isotopes

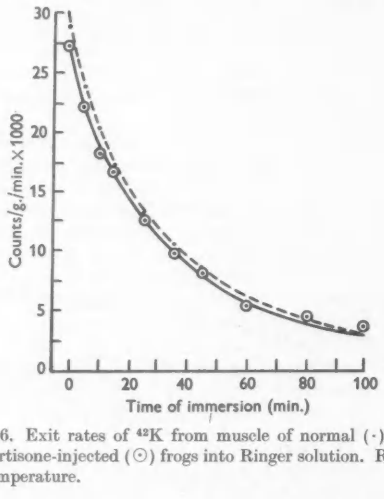

Fig. 6. Exit rates of ^{42}K from muscle of normal (·) and cortisone-injected (⊙) frogs into Ringer solution. Room temperature.

^{24}Na and ^{42}K. In measuring the entry rates of these ions, sartorii from normal and from cortisone-injected groups of frogs were immersed in Ringer solution containing the isotope and the radioactivity of the muscle (expressed as counts/g./min.) measured at short intervals. The results indicated that under the experimental conditions cortisone had no appreciable effect on the entry rates of these ions or on the exit rate into Ringer solution at room temperature. Fig. 6 illustrates a typical experiment with ^{42}K.

DISCUSSION

Phosphate esters of rat muscle

After adrenalectomy cortisone may (depending on the Na intake) be more effective than deoxycorticosterone in restoring the Na and K levels of plasma and muscle; similarly, deoxycorticosterone can act like cortisone in restoring the level of the hexose phosphates and of phosphocreatine and ATP, and in some degree the level of the fasting blood glucose (Harrison & Harrison, 1939). It appears that the classification into mineralo- and gluco-corticoids, as with cortisone and deoxycorticosterone is misleading. It is a question rather of dosage to produce equal effects and the proportions of the hormones required vary with the conditions and probably differ somewhat with each species.

On the other hand, the effect of cortisone on nitrogen metabolism and growth rate of young rats seems a practically qualitative difference compared with that of deoxycorticosterone, suggesting a unique effect of cortisone and related hormones in suppressing protein synthesis (Clark, 1950; Albright, 1943).

The marked reduction in the level of glucose 6-phosphate after adrenalectomy is related either to a less rapid formation or a more rapid disappearance. With a more rapid disappearance one would expect a marked increase of carbohydrate metabolism; in this respect it may be noted that Ingle & Nezamis (1948) have shown an increased rate of glucose utilization after adrenalectomy. With a slower formation after adrenalectomy it would seem as if, under the conditions examined, the effect is on the hexokinase system and both cortisone and deoxycorticosterone would appear as activators, cortisone being more effective than deoxycorticosterone. This is in contrast to results obtained by Colowick, Cori & Slein (1947) and by Krahl & Cori (1947) who have demonstrated by *in vitro* experiments that the hexokinase activity of muscles of diabetic rats can be inhibited by adrenal cortex extract and that the amelioration of diabetes by adrenalectomy is paralleled by a return of the glucose utilization of the diaphragm to normal.

The level of fructose 6-phosphate is also decreased after adrenalectomy but to a slightly lesser extent, the F 6-P/G 6-P ratio being 0·19 for the normal rat muscle and 0·29 for the adrenalectomized rat muscle; corresponding ratios for adrenalectomized rats treated with cortisone and deoxycorticosterone are 0·17 and 0·19, respectively. These values are somewhat low when compared with that obtained for phosphohexoseisomerase *in vitro* (0·35), possibly due to simultaneous utilization of F 6-P by phosphohexokinase and formation of G 6-P from glycogen and/or glucose.

While the hexose phosphate levels are much decreased, phosphocreatine and ATP are increased by adrenalectomy. The increase may be taken as statistically significant but relatively small compared with the total amounts present.

Sodium and potassium levels of rat muscle

From the results summarized in Table 2 it appears that such changes are secondary, or very largely secondary, to those of the plasma. When the plasma K falls, there is a secondary marked increase in the muscle Na, for which the following reason may be given. The fall of plasma K induces an increase in the membrane potential across the muscle fibre membrane and if this potential is already poised at

or near its maximum (Conway, 1947) a compensatory change of permeability may well occur with a net entrance of Na ions.

Renal effects. In turn the plasma changes themselves after deoxycorticosterone and cortisone injections arise from effects on the renal functions. This dependence of the tissue and plasma changes on the renal effects has previously been noted by Elkinton & Winkler (1944) and by Winkler, Smith & Hoff (1942) who found that deoxycorticosterone was without effect on the K metabolism of dogs following ligation of the ureters.

If the renal function with respect to Na and K is taken, in accordance with current views, as being reducible to an approximately constant filtration rate at the glomeruli combined with an approximately constant reabsorption rate of Na and of K, then the blood levels will depend on these two processes of filtration and reabsorption. If, for illustration, it be supposed that the reabsorption of K is inhibited by deoxycorticosterone, then deoxycorticosterone will cause a fall of plasma K, which in fact it does and especially with adequate Na intake. The effect on the K absorption under the circumstances (high-Na intake) need not appreciably affect the Na absorption, nor does the plasma Na level alter even after 20 days of deoxycorticosterone treatment, when at the same time the K level is greatly lessened.

After adrenalectomy the normal inhibition of K absorption produced by the cortical steroids is removed and the K level in the plasma markedly increases (cf. Harrison & Darrow, 1939). In turn it may be supposed that this back absorption of K inhibits the back absorption of Na, when the latter is low at the site (e.g. following low-Na intake), possibly by displacing Na from the absorption complex or mechanism. This assumption would serve at least to limit the number of hypothetical processes and receives support from the experiments of Miller (1922, 1926), Hoffman & Post (1933), Harrop (1936) and Kepler *et al.* (1948), who studied the relationships of Na and K intake in the diet to their retention and excretion.

On the other hand, if the diet is rich in Na the high concentration of Na resulting in the tubule lumen, at least below the level of the proximal convoluted tubule, may not only overcome the inhibition produced by this back absorption of K but in turn effectively inhibit it (Harrop, 1936; Wallace, 1948). Adrenalectomy should then produce but little effect on the K and Na levels of the plasma, and this is what in fact happens. Also it is a well-known fact that a high-Na intake can largely replace the action of the adrenal hormones on the cation pattern. Such a view as given above has the merit of correlating a number of leading facts.

The active transport of K^+ or Na^+ by the renal cells may be explained by the 'redox-pump' mechanism (Conway, 1951, 1952*a–c*), and the following paper deals with effects of the hormones on such a mechanism in yeast. It may be added that effects on membrane permeability to the free ions may well have a place. In the present paper permeability experiments were carried out on the isolated frog sartorius muscle using labelled ions but without any positive result. Since, as shown above, the Na and K effects on muscle are of a secondary kind, this is not surprising.

SUMMARY

1. Adrenalectomy causes a fall in the level of hexose phosphates and a rise in phosphocreatine and adenosine triphosphate of rat muscle. The hexose phosphate levels are restored towards normal by cortisone and by deoxycorticosterone, cortisone being more effective. Neither hormone has any effect on the levels of these substances in normal rats.

2. On a low-sodium intake both cortisone and deoxycorticosterone increase the plasma sodium and decrease the plasma potassium levels of normal rats, cortisone showing the greater effect. In a high-sodium intake neither hormone has any appreciable effect on the plasma sodium, even after a 20-day period of injection but both hormones lower the plasma potassium level and here deoxycorticosterone has a greater effect.

3. In adrenalectomized rats both hormones are effective in reversing the changes in plasma and muscle sodium and potassium; here again the relative effectiveness of the two hormones is dependent on the dietary sodium, cortisone being more effective with low-sodium intake. The muscle changes appear to be secondary, or largely secondary, to the plasma changes.

4. Cortisone is more effective than deoxycorticosterone in reducing the increased muscle-water content occurring after adrenalectomy.

5. Cortisone and deoxycorticosterone have opposite effects on the weight changes of normal and adrenalectomized rats, cortisone causing a marked decrease which is probably related to its effect in increasing urinary nitrogen excretion.

6. No apparent effect of either hormone on the permeability of frog sartorius muscle to ^{24}Na or ^{42}K was seen.

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The Effects of Cortisone, Deoxycorticosterone and other Steroids on the Active Transport of Sodium and Potassium Ions in Yeast

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During fermentation the yeast cell can actively excrete hydrogen ions and actively absorb potassium ions, the latter with a considerable amount of specificity (Conway & O'Malley, 1942, 1943, 1946; Rothstein & Enns, 1946).

Conditions can be arranged in which the exchange of the two ions results in an external pH of 1·5. Provided the external sodium-ion concentration is high (0·2 M-sodium citrate) and in the absence of any appreciable external concentration of potassium ions, sodium is also actively exchanged for hydrogen ions and to some extent for potassium ions. In this way after a single fermentation a yeast may be readily obtained which has a sodium content of the order of 50–100 m-equiv./kg. This cannot be readily washed out, but is in fact slowly and actively excreted on suspending in water and more rapidly if the suspending fluid contains potassium ions (Conway & Ryan, 1950; Conway, 1952*a*).

The various active transports have been interpreted by the theory of the 'redox pump' (Conway, 1951, 1952*b*, 1953), which is considered to function for the active extrusion of sodium ions from cells in general and so from muscle and nerve as well as from yeast and to function in the oxyntic cells of the stomach for the active secretion of hydrogen ions.

Because of the predominant role of the adrenal cortical hormones in the regulation of sodium and potassium exchanges in animals primarily in the renal nephrons, where active transports of sodium and potassium ions are taking place, the effect of these hormones on similar active transports in the yeast cell was considered worth investigating. Interesting results were quickly obtained and are described below.

METHODS

The production of a 'sodium yeast' (Conway & Moore, 1952). Yeast is fermented in 20 vol. of 5% (w/v) glucose containing 0·2 M-sodium citrate; under these conditions the cells rapidly accumulate Na up to a concentration of 100 m-equiv./kg. or more (i.e. 230 mg./100 g. yeast); this high concentration of Na is retained by the cell after washing several times with tap water. A portion of this 'sodium yeast', suspended in 20 vol. of a solution containing KCl and NaCl (usually 0·1 N-KCl and 0·1 N-NaCl was used), will be found to excrete Na^+ into the external fluid (Conway & Ryan, 1950). The excretion of Na is much reduced in the absence of K from the external solution and takes place then with some accompanying anion.

Hormone experiments. The effects of the addition of small quantities of crystalline steroids to the suspending fluid were studied; the active excretion and uptake of Na as well as the active uptake of K were investigated. In the examination of the active uptake of Na and K the yeast was allowed to ferment in 5% (w/v) glucose containing 0·2 M-sodium citrate or 0·1 M-potassium citrate. All of the hormones investigated in this manner (cortisone, deoxycorticosterone, testosterone, progesterone, oestrone, methylandrostenediol, dehydroepiandrosterone and 17-hydroxycorticosterone (Kendall's Compound F)) are only very slightly soluble in water and in salt solutions. For this reason ethanol at 5% (v/v) concentration was incorporated in the suspending fluid. The procedure adopted was to dissolve 5 mg. of the hormone in 5 ml. of pure ethanol and then add this to 95 ml. of the yeast suspension. The control suspension was treated similarly, omitting the 5 mg. hormone but including the 5 ml. ethanol. This incorporation of ethanol increased somewhat the solubilities of the hormones; nevertheless, the amounts going into solution were extremely small. Cortisone was used in the form of the acetate, as prepared by Merck and Co., Inc. and deoxycorticosterone as the acetate (DOCA) prepared by Organon Laboratories.

Na and K analyses were carried out on centrifuged, washed samples of the suspensions by means of the flame photometer.

RESULTS

Effects on the active excretion of sodium into 0·1 N-sodium chloride + 0·1 N-potassium chloride

Fig. 1 represents the excretion of Na by sodium yeast into 0·1 N-sodium chloride + 0·1 N-potassium chloride and the effects of cortisone and deoxycorticosterone on such excretion.

As can be seen the excretion of Na is markedly inhibited by deoxycorticosterone and to a smaller extent by cortisone. None of the other steroids mentioned above had any effect, nor had bile salts. Deoxycorticosterone always showed this inhibiting action; usually cortisone had a much smaller and scarcely appreciable effect.

Effects on the active excretion of sodium into tap water

Three experiments were carried out with sodium yeasts excreting into tap water (Na concn. 0·3 m-equiv./l., K concn. 0·01 m-equiv./l.). The mean Na

and K concentrations of the sodium yeasts were 211 and 210 mg./100 g. yeast, respectively, and after 20 min. in tap water the curve of Na loss flattened out, so that over the next 70 min. very little extra Na was lost.

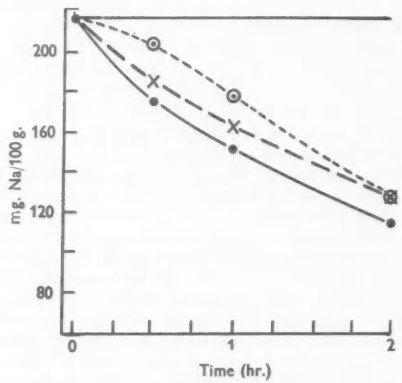

Fig. 1. Effects of cortisone and deoxycorticosterone on excretion of Na by Na-yeast into 0·1 N-NaCl + 0·1 N-KCl; the unbroken line represents the excretion of Na^+ into 0·1 N-NaCl with no external K. Untreated suspension (●); 5 mg. cortisone/100 ml. (×); 5 mg. deoxycorticosterone/100 ml. (⊙).

From 20 to 90 min. the mean loss of Na from the yeast was 75 ± 1·4 mg./100 g. yeast and in presence of deoxycorticosterone it was 79 ± 1·9 mg./100 g. These are the means for eight results with and without the hormone, the samples being taken at similar times. Here the hormone effect appears as a slight but not significant increased loss of Na.

The mean K values for the eight samples were 202 ± 0·9 and 201 ± 0·7 mg./100 g. with and without the hormone, which indicated no significant change.

From these results deoxycorticosterone does not appear to affect the excretion of Na when this occurs into tap water, i.e. in the virtual absence of external K^+.

Effects on the active uptake of potassium

Fig. 2 shows the inhibiting action of deoxycorticosterone on the active K uptake. Cortisone has practically no effect here. Of the other hormones 17-hydroxycorticosterone alone showed any definite result, and this was an activation and not an inhibition, the effect being relatively small but statistically significant. Owing to the small effect of 17-hydroxycorticosterone, the conditions of comparison had to be carefully standardized with respect to timing at all stages; it was found necessary to carry out the individual fermentations in centrifuge tubes, all the suspensions being then centrifuged and washed at the same time so that the fermentation times of the treated and untreated specimens were exactly the same. Table 1 gives the results of six experiments showing K uptake for control and

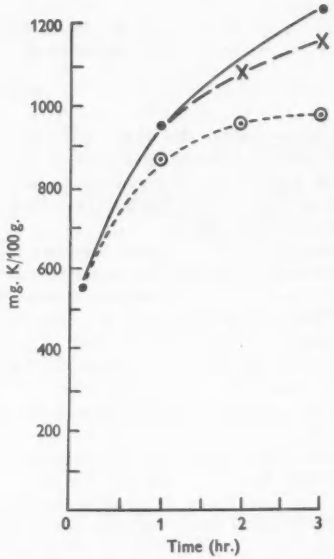

Fig. 2. Effects of cortisone and deoxycorticosterone on active uptake of K by yeast during fermentation in 0·1 M-potassium citrate. Untreated suspension (●); 5 mg. cortisone/100 ml. (×); 5 mg. deoxycorticosterone/100 ml. (⊙).

Table 1. *Effect of 17-hydroxycorticosterone on potassium uptake by yeast cells*

(Fermentation for 1 hr. in 20 vol. potassium citrate-glucose solution, as described in Methods section. The figures are increases in K content expressed as mg./100 g. yeast.)

Expt. no.	Untreated yeast	Yeast treated with 17-hydroxy-corticosterone	Difference due to hormone
1	294	326	+32
2	310	330	+20
3	270	300	+30
4	260	290	+30
5	250	330	+80
6	280	320	+40
Mean	277	316	+38·7 ± 8·7

17-hydroxycorticosterone suspensions during fermentation for 1 hr. In each case there was an increased uptake of K in the presence of the hormone, the mean of such increases being 38·7 ± 8·7 mg. K/100 g. yeast.

Fig. 3 shows the effect of 17-hydroxycorticosterone on K uptake during a 2 hr. fermentation period.

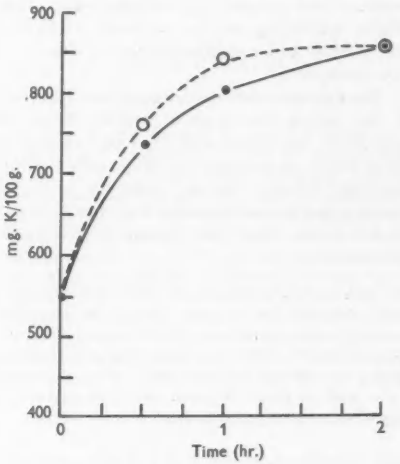

Fig. 3. Effect of 17-hydroxycorticosterone on active uptake of K by yeast during fermentation in 0·1 M-potassium citrate. Untreated suspension (●); 5 mg. 17-hydroxycorticosterone/100 ml. (○).

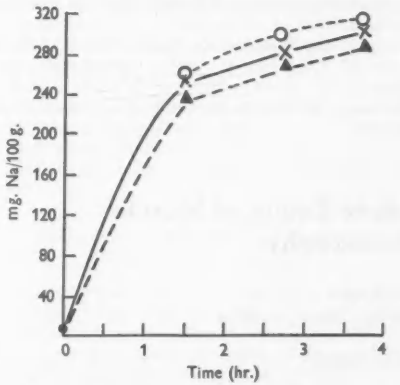

Fig. 4. Effects of cortisone, deoxycorticosterone and 17-hydroxycorticosterone on the active uptake of Na by yeast during fermentation in 0·2 M-sodium citrate. Untreated suspension and 5 mg. cortisone/100 ml. (×); 5 mg. deoxycorticosterone/100 ml. (▲); 5 mg. 17-hydroxycorticosterone/100 ml. (○).

The active uptake of sodium

Here the inhibiting effect of deoxycorticosterone again appears. Fig. 4 shows the results of an experiment in which Na is being actively absorbed from 0·2 M-sodium citrate. Cortisone was not found to have any significant effect. 17-Hydroxycorticosterone increased the uptake slightly.

In these experiments deoxycorticosterone produced a marked inhibition though the external concentration was as low as about $2{\cdot}5–3{\cdot}3 \times 10^{-5}$ M. The relative concentrations of deoxycorticosterone, cortisone, and 17-hydroxycorticosterone, when used in the external or suspending solutions, was roughly 1:2:5.

The concentrations at which deoxycorticosterone is effective on the active transport of Na or K is of a lower order than that of cyanide or azide on cell respiration.

DISCUSSION

Of the eight hormones studied for their effect on active Na and K transport across the yeast-cell membrane, three showed a significant effect, namely deoxycorticosterone, cortisone and 17-hydroxycorticosterone. The most pronounced was that of deoxycorticosterone. In a large number of experiments it never failed to show an inhibitory action, the effect on the active excretion of Na being the most pronounced.

Cortisone had a weaker effect and occasionally none could be shown but any exhibited was likewise of an inhibitory kind.

On the other hand, 17-hydroxycorticosterone showed a small but stimulating action on the active transports.

The nature of the inhibition produced by deoxycorticosterone

The inhibition of the sodium excretion could be considered either as a direct effect or an indirect one, in the latter case the primary action being on the K^+ entrance, with which the excreted Na^+ exchanges under the conditions of the experiments (no appreciable net H^+ exchange occurs). The fact that no effect of deoxycorticosterone is evident when the Na is excreted into tap water, indicates that the real effect on the Na excretion into a solution containing 0·1 M-KCl (as well as 0·1 M-NaCl) is on the K-carrier mechanism and this is in harmony with the view developed in the Discussion of the previous paper (Conway & Hingerty, 1953). An effect on free K^+ permeability seems a most unlikely cause of the inhibition as a tenfold change in the external concentration of K^+ outside the cell has scarcely any effect on the rate of active Na extrusion (Conway & Ryan, 1950).

Turning to the inhibition of the active K and Na uptake, the mechanism of such uptake may be considered to be the same, as by including only small amounts of K in the suspending fluid one can inhibit almost entirely the Na absorption. On the

other hand, the mechanism of active Na extrusion is not the same as that of K uptake, for the former is largely Na specific and the latter largely K specific.

Deoxycorticosterone may be taken then to inhibit indirectly active Na extrusion by its effect on the active K uptake or on the K carrier.

SUMMARY

1. Sodium ions accumulated by the yeast cell during fermentation in a medium containing a high concentration of sodium can be actively excreted into a medium containing potassium ions with which the sodium ions exchange. In the absence of external potassium, sodium is excreted much more slowly with an accompanying anion.

2. Of eight crystalline steroids (cortisone, deoxycorticosterone, testosterone, progesterone, oestrone, methylandrostenediol, dehydroepiandrosterone and 17-hydroxycorticosterone) investigated for a possible effect on such sodium and potassium exchanges in the yeast cells, as well as on active potassium uptake during fermentation only three (deoxycorticosterone, cortisone and 17-hydroxycorticosterone) showed significant effects; deoxycorticosterone showed the most pronounced effect, namely an inhibitory action on the active transport of sodium and potassium; cortisone also showed a slight inhibiting action whereas 17-hydroxycorticosterone had a small stimulating effect on the active transport.

3. Deoxycorticosterone does not inhibit appreciably the active excretion of sodium from yeast suspended in tap water and it is concluded that its marked effect on sodium excretion into a solution containing 0·1 M-potassium chloride is on the potassium-carrier mechanism which has been interpreted in terms of a 'redox pump' (Conway, 1951, 1952*b*, 1953).

The authors wish to thank the Rockefeller Foundation for Medical Research for a grant in aid; also the Medical Research Council of Ireland, and in particular Prof. H. Moore, University College, Dublin, for his kindness in supplying us with the cortisone used in these experiments. We also wish to thank Organon Laboratories Ltd. for a supply of the other crystalline steroids used.

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The Separation of the Phosphate Esters of Muscle by Paper Chromatography

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Knowledge about the phosphorus metabolism of muscle depends on the separation and determination of the phosphate esters in muscle extracts. In the early work, such as that done by Eggleton & Eggleton (1929), methods are described whereby the adenosine polyphosphate, creatine phosphate, orthophosphate and monophosphate ester fractions can be separated and determined. These methods have given valuable information about the large changes in these esters in muscle after long periods of activity, but they are difficult to use, particularly for investigating short periods of activity in which the changes are small.

In this work the chromatographic method described by Hanes & Isherwood (1949), which has not so far been used for the esters in muscle extracts, has been examined. The esters in frog muscle have been compared with those in tortoise muscle and, to compare this method with the others, some of the early work on the changes during long periods of

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activity have been repeated. The method has been used in an attempt to obtain with tortoise muscle, following a suggestion of Hill (1949, 1950*b*), some information about the changes in the esters during short periods of activity.

EXPERIMENTAL

Chromatographic reference compounds

Adenosine triphosphate (*ATP*). Two samples were used. One, which gave a single spot on the chromatograms, was a gift from Dr J. Ottaway of the Biochemistry Department, University College, London. The other, a commercial one, described as 95% pure, was used in the recovery experiments (Table 2). It was found to contain small amounts of adenosine diphosphate (ADP) and orthophosphate. Other samples were examined but were found to contain up to four fractions.

Adenosine diphosphate. This was made from commercial ATP by the method of Lohmann (1935). It gave a single spot in the solvents used; 43% of the P was split off as orthophosphate on heating with N-H_2SO_4 for 10 min. at 100°.

Adenosine 5′-phosphate (*AMP*). This was a synthetic sample.

Creatine phosphate. This was a synthetic sample made by the method of Zeile & Fawaz (1938). It gave a single spot in the *n*-propanol/ammonia solvent. In the *tert.*-butanol/picric acid solvent it was unstable and usually gave a streak.

Orthophosphate, pyrophosphate, tripolyphosphate and hexametaphosphate. These were commercial samples.

Fructose 6-phosphate and fructose 1:6-diphosphate. These were gifts from Mr C. W. Parr of the Biochemistry Department, University College, London. Both gave single spots in the solvents used.

2:3-Diphosphoglyceric acid. This was a gift from Prof. M. Maizels. It was found by chromatography to contain about 20% of monophosphoglyceric acid.

Ethanolamine phosphate. This was a gift from Dr S. P. Datta of the Biochemistry Department, University College, London.

Technique for stimulation of muscle and preparation of extract

The muscles used. These were the gastrocnemius of the frog (*Rana temporaria*), the iliofibularis, the extensor iliotibialis and the sartorius of the tortoise (*Testudo graeca*), and the retractor pharynx muscle of the snail (*Helix pomatia*). The iliofibularis and extensor iliotibialis were once called the biceps cruris and the rectus femoris and are so described by Owen (1866, fig. 151, nos. 103 and 99). The sartorius is described as such by Owen (1866, fig. 151, no. 106). The weight of these muscles was usually between 300 and 1500 mg., except that of the snail which was 17 mg. In experiments in which a stimulated muscle was compared with an unstimulated one, the two pair muscles from the left- and right-hand sides of the same animal were used.

Fixation and stimulation of muscles. The muscles were fixed by freezing. Fixation in the stimulated state was done by suddenly immersing them in a mixture of acetone and solid CO_2. This sudden freezing causes stimulation (see Lundsgaard, 1950) and makes it possible to fix the muscles at the beginning of activity. Muscles which were fixed after a period of activity were frozen suddenly while the electrical stimulation was still being given. Frog muscles which have been frozen suddenly are fully contracted, but tortoise muscles which have not first been stimulated by other means are usually not, probably because they freeze before they have time to shorten.

Fixation in the unstimulated state was carried out by slow freezing (as suggested by Lundsgaard, 1950). The muscles were hung in a conical flask so that they did not touch the walls and then the flask was placed in a Dewar vessel containing solid CO_2. The muscles took about 30 min. to freeze and did not show any sign of stimulation as long as they did not touch the cold sides of the flask. The protein residue from muscles fixed in this way was less opaque than that from those fixed by sudden freezing.

Preparation of muscle extracts. The muscles were broken up at about −70° and the powder dropped into 2–5 ml. of a 5% solution of trichloroacetic acid (TCA) in acetone (also at about −70°). Any lumps were broken up with a glass rod and the suspension was allowed to stand for about 1 hr. This was done to inactivate as much of the enzyme protein as possible at a low temperature so that spurious changes in the phosphate esters were lessened. The suspension was then slowly warmed up to room temperature with constant stirring. When this was reached the acetone was evaporated off, about 1 ml. of water was added and the powdered muscle extracted with the now aqueous TCA for about 1 hr. at 0°. Then the whole was washed into a graduated centrifuge tube and made up to the appropriate volume (1·5–4·0 ml. depending on the weight of the muscles).

Removal of calcium and magnesium from the extracts. Small amounts of calcium and magnesium in the extracts, which would spoil the chromatograms, were turned into soluble complexes with ethylenediaminetetraacetic acid (Walker & Warren, 1951). Usually 1 ml. of protein-free muscle extract was put into a graduated tube and 50 mg. of ethylenediaminetetraacetic acid added for every g. of muscle to which the amount of extract corresponded. It was then made alkaline with concentrated ammonia solution to lessen the breakdown of creatine phosphate, the total volume being brought to 1·1 ml. The ethylenediaminetetraacetic acid did not greatly change the distances travelled by the esters.

Chromatography

Choice of solvents. The most useful of the solvents suggested by Hanes & Isherwood (1949) have been found to be *n*-propanol/ammonia/water (60 ml. *n*-propanol:30 ml. conc. ammonia solution:10 ml. water) and *tert.*-butanol/picric acid/water (80 ml. *tert.*-butanol:20 ml. water:4 g. picric acid). Some chromatograms were run in a solvent of ethyl acetate/pyridine (water-poor phase from 100 ml. ethyl acetate:45 ml. pyridine:100 ml. water) but this was abandoned because orthophosphate often gave two spots.

The distances that spots of some phosphate esters and polyphosphoric acids travel in the two suggested solvents are given in Table 1, these distances being given in terms of that reached by orthophosphate. This is a more useful standard than the R_F value, particularly with the *n*-propanol/ammonia solvent, where the R_F values are difficult to measure because some of the solvent must be taken up on a pad of filter paper fixed to the bottom of the chromatogram. The values in Table 1 can be turned into the R_F values if they are multiplied by the R_F value for ortho-

phosphate. This is about 0·2 in *n*-propanol/ammonia and 0·7 in *tert.*-butanol/picric acid.

ADP travels the same distance as orthophosphate in the *n*-propanol/ammonia solvent and about the same distance as ATP in the *tert.*-butanol/picric acid solvent. It can be separated on a two-dimensional chromatogram in which the two solvents are used successively. ATP, orthophosphate, the creatine phosphate plus monophosphate ester fraction, the diphosphate esters, pyrophosphate and tripolyphosphate are also separated on a two-dimensional chromatogram, but the individual mono- and di-phosphate esters are not.

Table 1. *Distances travelled by some phosphate esters in the chromatographic solvents used*

Substance	Distance travelled (in terms of that travelled by orthophosphate)	
	n-Propanol/ ammonia	*tert.*-Butanol/ picric acid
Monophosphoglycerate	1·7	0·75
Adenosine 5′-phosphate	1·5	0·78
Creatine phosphate	1·5	0·56
Ethanolamine phosphate	1·4	0·62
Fructose 6-phosphate	1·3	0·67
Adenosine diphosphate	1·0	0·20
Adenosine triphosphate	0·6	0·13
Pyrophosphate	0·6	0·48
2:3-Diphosphoglycerate	0·5	0·75
Fructose 1:6-diphosphate	0·5	0·67
Tripolyphosphate	0·5	0·28
Hexametaphosphate	Streaks 0·0–0·6	Streaks 0·00–0·50
Unknown *A*	3·5	—
Unknown *B*	1·7	0·20
Unknown *C*	0·9	0·40
Unknown *D*	0·6	0·60
Unknown *E*	0·5	0·10
Unidentified ester in snail muscle extract	1·0	0·65

Running of the chromatograms. Successive 5 μl. volumes of the extract (total 50–200 μl.) were put on the paper with a micropipette, the moisture being removed in a current of air. The paper was Whatman no. 4 filter paper which had been washed with 2 N-acetic acid as suggested by Hanes & Isherwood (1949). The chromatograms were run at room temperature, usually for 16 hr. in *n*-propanol/ammonia and 8–10 hr. in *tert.*-butanol/picric acid.

Development of the chromatograms. The positions of the esters on the paper were found by spraying with the mixture suggested by Hanes & Isherwood (1949) (5 ml. 60% perchloric acid; 25 ml. 4% ammonium molybdate; 10 ml. N-HCl; water to 100 ml.), followed by heating in an oven at 85° for 10 min. and treatment with H_2S, the esters appearing as blue spots.

Two other tests could be done. When the chromatogram was sprayed with the reagent, orthophosphate appeared at once and creatine phosphate after 1 min. as yellow spots due to the formation of phosphomolybdic acid. The adenosine phosphates could be found by clipping to the chromatogram a piece of filter paper which had been dyed with proflavine and then looking at an ultraviolet lamp through the two layers, the radiation passing first through the chromatogram. The areas of the chromatogram in which the adenosine phosphates were present absorbed the radiation so that the proflavine in the adjacent paper did not fluoresce and the adenosine phosphates appeared as dark spots on a fluorescent background. Neither of these tests could be done with chromatograms run in the *tert.*-butanol/picric acid solvent because the acid interferes.

Quantitative determinations. A standard area of the chromatogram, which included the spot of the ester to be determined, was cut out, put into a 100 ml. conical Pyrex flask and 5 ml. of M-K_2SO_4 solution which contained H_2SO_4 and a trace of $CuSO_4$ added. The whole was then evaporated to dryness, 1–2 ml. of conc. H_2SO_4 added and the paper oxidized by wet ashing, the oxidation being completed in about 5 min. The excess H_2SO_4 was then boiled off and the molten potassium hydrogen sulphate cooled and allowed to solidify. The solid was dissolved in water and heated at about 95° for 15 min. to turn all the P into orthophosphate. The sample was then transferred to a separating funnel, its total volume brought to 10 ml. and the P determined by the method of Berenblum & Chain (1938). A colorimeter which had been made by Dr E. J. Harris, the design of which was based on that described by Humphrey & Harris (1948), was used. The amount of K_2SO_4 added in this method is such that the final solution, when diluted to 10 ml., contains enough hydrogen sulphate to bring the hydrogen-ion concentration within the limits given by Berenblum & Chain, namely 0·1–2·0 N.

Other analytical methods

Determination of creatine phosphate and the monophosphate ester fraction. Creatine phosphate is not separated from the monophosphate esters on the chromatograms and determination of the P in the appropriate spot gives the sum of these compounds. If some of the extract is treated with 5% ammonium molybdate and N-H_2SO_4 for 30 min. at 20°, subsequent determination of the P gives a value for the sum creatine phosphate + orthophosphate. The value obtained for orthophosphate from the chromatogram can be subtracted from this to give a value for creatine phosphate, which can then be subtracted from the value obtained from the chromatogram for creatine phosphate + monophosphate esters to give one for the monophosphate esters.

Determination of the protein phosphorus. In most of the experiments in which tortoise muscles were used, the amount of P attached to the muscle protein was determined. The residue, after the first extraction with TCA, was washed five times with 5% TCA. The P in the residue was then determined by the method used for the P in the chromatogram spots.

RESULTS

Tests of the chromatographic method

The method was tested directly in two experiments. In the first, amounts of ATP and orthophosphate in the proportions found in normal muscle were put on the paper; in the second experiment amounts of orthophosphate, fructose 6-phosphate and fructose 1:6-diphosphate in the proportions found in muscles which have been poisoned by monoiodoacetate and then fatigued were used. The chromatograms were run in one direction in *n*-propanol/ammonia and in

the other in *tert.*-butanol/picric acid. The spots were then cut out and analysed for phosphorus. The results are given in Table 2 which shows that the values obtained agree with the known amounts put on the paper within 10%.

Table 2. *The recovery from two-dimensional chromatograms of mixtures of phosphate esters*

	Amount recovered from chromatogram (μg. P)			Amount applied (μg. P)
Expt. 1	*A*		*B*	
Orthophosphate	12·0		11·5	12·0
ATP	8·4		8·2	8·7
Total	20·5		19·7	20·7
Expt. 2	*A*	*B*	*C*	
Orthophosphate	20·4	21·2	20·4	19·1
Fructose 6-phosphate	12·0	10·7	13·6	13·5
Fructose 1:6-diphosphate	21·3	19·6	22·7	22·7
Total	53·7	51·5	56·7	55·3

Table 3. *Total phosphorus recovered from chromatograms of muscle extracts*

Type of muscle	Amount recovered from chromatogram (μg. P/g. of muscle)	Amount present in extract by direct estimation (μg. P/g. of muscle)
Frog	1526	1517
	1692	1609
Tortoise	901	942
	876	850
	1001	1073
	1192	1212

Comparisons of the total phosphorus in the muscle extracts with that recovered from the chromatograms are given in Table 3, which shows that the values agree to within 7–8%. The total orthophosphate in the extracts after 10 min. acid hydrolysis has been compared with the combined value for the labile phosphorus of ATP and ADP, orthophosphate and creatine phosphate and found to agree to within 15%. If the extracts contained radioactive orthophosphate, their activity agreed with that of the orthophosphate spot to within 10%.

The phosphate esters in muscle extracts compared with known esters

One-dimensional chromatograms of extracts of normal muscle show three well-defined spots. In the *n*-propanol/ammonia solvent these correspond to ATP, ADP + orthophosphate and creatine phosphate + monophosphate esters, and in the *tert.*-butanol/picric acid solvent to ATP + ADP, creatine phosphate + monophosphate esters and orthophosphate. Two-dimensional chromatograms, in which the first solvent is *n*-propanol/ammonia and the second *tert.*-butanol/picric acid, show four spots. These correspond to ATP, ADP, orthophosphate and creatine phosphate + monophosphate esters. The ADP spot is usually faint. Its separation from ATP is sometimes unsatisfactory and then they must be determined together. Chromatograms of extracts of muscles which have been poisoned with monoiodoacetate and then fatigued show a spot corresponding to the diphosphate esters. This is not found with normal muscle, but analysis of the appropriate area of the chromatogram usually shows the presence of some phosphorus.

Some other fractions, which have not been identified, have been found, mainly in tortoise muscles. The two principal ones have been called unknowns *A* and *B* and are stable to the perchloric acid/ammonium molybdate reagent. With the present methods they can only be found by analysis of the appropriate area of the chromatogram. Three other fractions, which were shown up by the perchloric acid/ammonium molybdate reagent, were found in six muscles obtained from the same tortoise. These have been called unknowns *C*, *D* and *E*. The distances travelled by the unknowns on the chromatograms are given in Table 1.

Comparison of the amounts of the phosphate esters in frog and tortoise muscle

A histogram is given in Fig. 1 for the amounts of ATP and creatine phosphate + orthophosphate in the muscles of frog and tortoise. The frog muscles usually contain larger amounts than those of tortoise. A single value of 156 μg. phosphorus/g., which falls in the middle of the range of those for tortoise muscles, was obtained for the amount of ATP in the retractor pharynx muscle of the snail, which has about the same speed as tortoise muscle. There was no spot corresponding to creatine

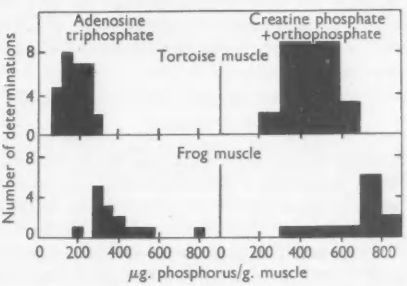

Fig. 1. Histogram of the ATP and creatine phosphate + orthophosphate contents of frog and tortoise skeletal muscle.

Table 4. *Comparisons of the phosphate ester contents of pair frog muscles, one muscle having been stimulated, the other not*

Period of electrical stimulation (sec.)		Expt. 1 15	Expt. 2 10	Expt. 3 120	Expt. 4 Stimulated and fixed by sudden freezing
		Phosphate esters (μg. P/g. of muscle)			
ATP	Stimulated	437	299	288	392
	Unstimulated	504	294	284	363
	Difference	−67	+5	+4	+29
ADP	Stimulated	56	60	—	40
	Unstimulated	59	17	—	58
	Difference	−3	+43	—	−18
Creatine phosphate*	Stimulated	28	202	252	500
	Unstimulated	281	433	413	580
	Difference	−253	−231	−161	−80
Orthophosphate†	Stimulated	650	496	481	274
	Unstimulated	446	328	297	215
	Difference	+204	+168	+184	+59
Monophosphate esters	Stimulated	149	—	—	—
	Unstimulated	70	—	—	—
	Difference	+79	—	—	—
Diphosphate esters	Stimulated	6	19	—	2
	Unstimulated	3	21	—	0
	Difference	+3	−2	—	+2

* Expts. 2 and 3 include monophosphates.
† The results given in Expt. 3 are for a one-dimensional chromatogram run in *n*-propanol/ammonia. In this solvent ADP is not separated from orthophosphate. The other results are all for two-dimensional chromatograms.

phosphate on the chromatogram of this snail muscle, but a compound was present which had travelled the same distances as unknown *C*.

Comparison of stimulated and unstimulated frog muscles

Results from these experiments are given in Table 4. In Expts. 1–3 the stimulated muscles were tetanized with shocks from an induction coil before being fixed by sudden freezing. The changes found in them, namely a decrease in creatine phosphate and increases in orthophosphate and the monophosphate esters, are the same as those found in the early work on muscle. In Expt. 4 the stimulated muscle was both fixed and stimulated by sudden freezing. Under these conditions, the closest approximation to a single twitch which is possible with frog muscle, the stimulated muscle contained less creatine phosphate and more orthophosphate than the unstimulated one. In none of the experiments was there any significant breakdown of ATP into ADP.

Experiments with frog muscles poisoned with monoiodoacetate

In these experiments pair frog gastrocnemius muscles were poisoned by being soaked for 3 hr. in a Ringer solution containing 2×10^{-4} M-monoiodoacetate. It was necessary to take care in dissecting out the muscles because if they were damaged they developed rigor mortis after about 30 min. soaking. After soaking, one muscle was fixed without stimulation by slow freezing. The other was stimulated with an induction coil and made to carry out a rapid series of single twitches after about 150 of which it ceased to respond (cf. Lundsgaard, 1930*a*). It was then fixed before it had started to develop rigor.

The results of two of these experiments are given in Table 5. In two other experiments, only the ATP and diphosphate ester fractions were determined and the following changes were found, the amounts being expressed as μg. phosphorus/g. In Expt. 3 the ATP decreased from 347 to 247 and the diphosphate esters increased from 20 to 391 on stimulation; in Expt. 4 the ATP decreased from 203 to 111 and the

Table 5. *Comparison of the phosphate ester contents of pair frog muscles poisoned with monoiodoacetic acid, one muscle having been stimulated to inexcitability but not to rigor, the other unstimulated*

(From gastrocnemius muscles soaked 3 hr. in Ringer solution + 2×10^{-4} M-iodoacetate.)

	Phosphate esters (μg. P/g. of muscle)					
	Expt. 1			Expt. 2		
	Stimulated	Unstimulated	Difference	Stimulated	Unstimulated	Difference
ATP	200	365	−165	60	238	−178
ADP	129	96	+33	135	57	+78
Creatine phosphate	116	351	−235	7	402	−395
Orthophosphate	361	399	−38	183	256	−73
Monophosphate esters	58	191	−133	—	—	—
Diphosphate esters	368	38	+330	224	38	+186
Unknown *A*	167	144	+23	—	—	—
Unknown *B*	126	109	+17	8	0	+8

Table 6. *Comparisons of the phosphate ester contents of pair tortoise muscles, both muscles being treated identically*

	Type of muscle								
	Iliofibularis			Extensor iliotibialis			Sartorius		
Fraction	Left	Right	Diff.	Left	Right	Diff.	Left	Right	Diff.
	Phosphorus contents, expressed as μg. P/g. of muscle								
Total extractable P	1100	1099	1	989	1172	183	1074	1148	74
Protein P	—	—	—	224	223	1	205	158	47
	Phosphorus contents, expressed as % of total extractable P								
ATP	20·6	22·2	1·6	27·1	25·9	1·2	22·4	25·2	2·8
ADP	6·3	7·0	0·7	1·6	2·3	0·7	7·0	5·7	1·3
Creatine phosphate	31·1	29·6	1·5	30·3	32·7	2·4	21·0	22·0	1·0
'High-energy' P*	48·0	47·9	0·1	49·2	51·2	2·0	39·4	41·6	2·2
Orthophosphate	25·5	24·8	0·7	28·4	25·5	2·9	31·0	29·8	1·2
Monophosphate esters and adenylic acid	0·0	0·0	0·0	0·0	0·0	0·0	2·3	2·3	0·0
Diphosphate esters	4·2	5·0	0·8	0·0	0·0	0·0	0·7	0·6	0·1
Unknown *A*	8·1	6·5	1·6	6·8	7·7	0·9	8·8	8·2	0·6
Unknown *B*	4·2	5·0	0·8	5·8	6·0	0·2	6·8	6·2	0·6

* Sum of creatine phosphate + two-thirds ATP + one-half ADP.

diphosphate esters increased from 0 to 306. The increase in the diphosphate ester fraction observed in these experiments agrees with Lundsgaard's (1930*b*) conclusion that the esters which accumulate in these poisoned muscles consist largely of hexose diphosphate.

Comparisons of the phosphate ester contents of pair tortoise muscles treated alike

Pairs of tortoise muscles from the right and left sides of the same animal often differ in weight, sometimes by as much as 20%. To see if this difference was associated with any serious difference in the amounts of the phosphate esters in the muscles of a pair, some experiments were done in which both muscles were fixed in the unstimulated state by slow freezing. The results are given in Table 6. This shows that in two experiments a difference was found in the total amount of extractable phosphorus, which increased the differences in the amounts of the individual esters if these were expressed as μg. phosphorus/g. The amounts of the esters are more satisfactorily expressed as a percentage of the total extractable phosphorus, and Table 6 shows that if this is done, the differences between two muscles of a pair are not more than 10%.

Comparisons of stimulated and unstimulated tortoise muscles

In these experiments, following a suggestion of Hill (1949, 1950*b*), tortoise muscles have been used in an attempt to follow the changes in the phosphate esters during the early stages of activity. These muscles, being much slower than those of frog, are easier to fix at a given stage of contraction.

Muscles stimulated by sudden freezing. In these experiments the muscles were first cooled to 0°. One was stimulated and fixed at an early stage of contraction, when little or no shortening had occurred, by sudden freezing and the other was fixed without stimulation by slow freezing. The results of five of these experiments are given in Table 7. In an incomplete experiment, Expt. 6, the following

Table 7. *Changes in the phosphate ester contents of pair tortoise muscles, one muscle having been fixed and stimulated by sudden freezing, the other fixed by slow freezing*

	Type of muscle						
	Expt. 1. Ilio-fibularis	Expt. 2. Ilio-fibularis	Expt. 3. Ilio-fibularis	Expt. 4*. Extensor ilio-tibialis	Expt. 5. Sartorius		
	Length when frozen (% of resting length)						
Stimulated (frozen suddenly)	79	100	100	93	100		
Unstimulated (frozen slowly)	100	100	100	100	100		
	Change in phosphate esters on stimulation					Mean content	
Fraction	Difference (stimulated − unstimulated)					Mean change	Stimulated	Unstimulated
	Expressed as μg. P/g. of muscle							
Total extractable P	−89	+25	−69	+120	−191	−40	967	1007
Protein P	—	−2	+11	+32	−11	−3§	237§	240§
	Expressed as % of total extractable P							
ATP†	−5·0	−5·2	+3·2	−2·8	+0·6	−1·8	15·9	17·7
ADP	−0·9	−1·3	—	+0·2	−0·3	−0·6	4·8	5·4
Creatine phosphate	−6·0	+0·1	−2·5	−0·8	−2·3	−2·3	15·1	17·4
'High-energy' P‡	−9·8	−4·0	−0·4	−2·6	−2·1	−3·8	28·1	31·9
Orthophosphate	+9·9	+3·6	−4·7	−4·0	+0·5	+1·1	32·2	31·1
Monophosphate esters and adenylic acid	+3·9	+1·6	+3·4	+1·2	−0·4	+1·9	5·6	3·7
Diphosphate esters	−0·1	0·0	+2·3	−0·2	+0·6	+0·5	1·5	1·0
Unknown *A*	−2·9	−1·5	+2·3	+5·8	+2·2	+1·2	12·8	11·6
Unknown *B*	−0·6	+2·7	−3·9	+0·6	−0·2	−0·3	10·5	10·8
Unknown *C*	+0·2	—	—	—	0·0	+0·1	2·3	2·2
Unknown *D*	+1·4	—	—	—	0·0	+0·7	2·6	1·9
Unknown *E*	+0·2	—	—	—	−1·0	−0·4	1·9	2·3

* In this experiment the stimulated muscle was stimulated electrically just before being frozen.
† Expt. 3 includes ADP.
‡ Sum of two-thirds ATP + one-half ADP + creatine phosphate.
§ These three means include the results of one additional experiment which is described in the text.

differences, expressed as μg. phosphorus/g., were found (value for stimulated muscle − value for unstimulated muscle): protein phosphorus, −42; ATP, −37; ADP, −12; creatine phosphate, −40; orthophosphate, −154; diphosphate esters, −4. The values for the protein phosphorus obtained in this experiment have been included in the means given in Table 7, but the other values have not because they cannot be expressed as a percentage of the total extractable phosphorus.

The results are somewhat erratic. The only clear-cut change on stimulation was a decrease in the 'high-energy' phosphate fraction (sum of two-thirds ATP + one-half ADP + creatine phosphate). In all experiments except one there was a decrease in creatine phosphate and ADP and an increase in the monophosphate esters. There was little sign of a change in the protein phosphorus. The decreases in ATP observed in Expts. 1, 2 and 6 are outside the usual variability found between pair muscles, whereas the changes in Expts. 3–5 are not, and many more experiments will have to be done to decide whether there is a breakdown of the ATP of tortoise muscle in this kind of experiment.

Muscles stimulated electrically. The muscles were cooled to 0°. One was stimulated with condenser discharges at the rate of 6/sec. and after 5 sec., when it had just become fully contracted, it was fixed by sudden freezing while the stimulus was still being applied. The other, which was not stimulated, was fixed by slow freezing. The results of three experiments are given in Table 8. In two incomplete experiments, Expts. 4 and 5, the stimulated muscles were stimulated at a rapid rate with an induction coil, in Expt. 4 for 2 sec. and in Expt. 5 for 5 sec. The changes (value for stimulated muscle − value for unstimulated muscle, in μg. phosphorus/g.) were: in Expt. 4: protein phosphorus, −27; ATP, +12; ADP, −11; creatine phosphate, −8; orthophosphate, +62; diphosphate esters, +4; in Expt. 5: protein phosphorus, +8; ATP + ADP, +35; creatine phosphate, −56; orthophosphate, +70; diphosphate esters, +20. The values for the protein phosphorus obtained in these two experiments have

Table 8. *Changes in the phosphate ester contents of pair tortoise muscles, one muscle having been stimulated electrically for 5 sec. before fixation, the other not*

	Type of muscle		
	Expt. 1. Ilio-fibularis	Expt. 2. Extensor ilio-tibialis	Expt. 3. Sartorius
Length when frozen (as % of resting length)			
Stimulated	40	57	63
Unstimulated	100	100	100
Work done by stimulated muscle (g.cm.)	51	17	10

Fraction	Change in phosphate esters on stimulation: Difference (stimulated – unstimulated)			Mean change	Mean content: Stimulated	Mean content: Unstimulated
	Expressed as μg. P/g. of muscle					
Total extractable P	−111	−39	−79	−76	773	849
Protein P	+6	−11	−23	−11†	220†	231†
	Expressed as % of total extractable P					
ATP*	−0·2	+2·0	−2·8	−0·6	26·7	27·1
ADP	—	+0·9	—	+0·9	7·2	6·3
Creatine phosphate	−7·8	−4·7	−7·6	−6·7	14·8	21·5
'High-energy' P‡	−7·8	−2·8	−9·4	−6·7	36·1	42·8
Orthophosphate	+1·6	−0·8	+3·3	+1·3	31·4	30·1
Monophosphate esters and adenylic acid	+1·7	+4·6	+4·8	+3·7	3·7	0·0
Diphosphate esters	−0·1	0·0	−0·3	−0·2	1·7	1·9
Unknown *A*	+2·6	−2·4	+0·2	+0·2	11·7	11·5
Unknown *B*	+2·2	+0·6	+2·5	+1·8	7·6	5·8

* Expts. 1 and 3 include ADP.
† These three means include the results of two additional experiments which are described in the text.
‡ Sum of two-thirds ATP + one-half ADP + creatine phosphate.

been included in working out the means given in Table 8, but the other values have not since they cannot be expressed in terms of the total extractable phosphorus.

The results show that after 5 sec. stimulation of tortoise muscle, creatine phosphate and the 'high-energy' phosphate fraction had decreased and the monophosphate esters and unknown *B* had increased. Orthophosphate had increased in all experiments except one. There was little sign of changes in the other fractions.

DISCUSSION

This work has shown that if the chromatographic method of Hanes and Isherwood is used in conjunction with other methods, the phosphate esters in muscle extracts can be separated into ATP, ADP, creatine phosphate, orthophosphate, the monophosphate ester fraction, the diphosphate ester fraction and up to five unidentified fractions, and quantitative values for these obtained.

The values for frog muscle are in good agreement with those obtained by Eggleton & Eggleton (1929), Lundsgaard (1930*b*) and Causey & Harris (1951) with other methods, which fact gives confidence in the chromatographic method. The values show that there is more ATP and creatine phosphate + orthophosphate in frog muscle than in tortoise muscle, and this comparison of a fast striated muscle with a slow one shows the same trend as those of striated muscles with the slower smooth muscles. Thus Walaas & Walaas (1950) have shown that the slow unstriated uterine muscles of rats and rabbits contain much less ATP and creatine phosphate than the fast striated skeletal muscles.

The values for ADP in frog and tortoise muscle show that this usually forms 10–20 % of the pyrophosphate fraction. This is higher than the values of 1–5 % obtained by Bailey (1949) with rabbit muscle by enzymic analysis. No inorganic pyrophosphate, tripolyphosphate or metaphosphate was found in the extracts with the possible exception of a few of those of tortoise muscle, the unknown *D* in which may have been pyrophosphate.

The unidentified ester found in snail muscle was probably arginine phosphate but this has not been confirmed. The identity of the other unknown esters and their function in muscle is obscure. Cori & Cori (1931) state that about 70 μg./g. of the phosphorus in rat-muscle extracts does not correspond to known phosphate esters and it is possible that this consists of the unknown esters now detected.

Current theories of muscular contraction postulate that the hydrolysis of ATP to ADP and ortho-

phosphate provides the energy for mechanical work (see, for example, Baldwin, 1952). To demonstrate this an increase in orthophosphate and either a decrease in ATP or an increase in ADP must be shown in the stimulated muscle, preferably in the same experiment. There was no such direct evidence, except in muscles fixed after exhaustion, until recently when experiments on the changes early in activity were published by Lundsgaard (1950) which suggested a decrease in the ATP + ADP fraction and by Mommaerts & Rupp (1951) which suggested a decrease in ATP and increases in ADP and AMP.

In the present work very little evidence for these changes has been obtained. The experiments in which stimulated and unstimulated unpoisoned frog muscles were compared show, in the stimulated muscle, only the decrease in creatine phosphate and the increases in orthophosphate and the monophosphate esters found by earlier workers (see, for example, Eggleton & Eggleton, 1929; Cori & Cori, 1934). Those in which exhausted and unstimulated frog muscles which had been poisoned with monoiodoacetate were compared show the changes found by Lundsgaard (1930*b*), namely decreases in creatine phosphate and the ATP + ADP fraction and an increase in the diphosphate ester fraction on exhaustion. Of the components of the ATP + ADP fraction, the ATP decreased and the ADP increased, but the results do not provide evidence for the hydrolytic breakdown of ATP during the exhaustion of the poisoned muscles because all the phosphorus which disappears from the ATP and creatine phosphate fractions reappears in the diphosphate ester fraction and not as orthophosphate. There were still appreciable amounts of ATP in the exhausted muscles, and their exhaustion cannot be due to the complete conversion of their supply of ATP into ADP which Lundsgaard's figures suggest.

The continued presence of ATP is no doubt due to the intervention of myokinase, which converts ADP into ATP and AMP. An equilibrium constant for this change in exhausted muscles can be obtained if the amount of AMP in them is assumed to be equal to the amount of ATP which has disappeared, allowance being made for the ATP which has gone into the ADP fraction. The constants calculated from Expts. 1 and 2 of Table 5 for $\frac{[ATP]\,[AMP]}{[ADP]^2}$ are 0·6 and 0·1. These are of the same order as the value 0·444 which has been obtained by Eggleston & Hems (1952) with the isolated enzyme.

The experiments with tortoise muscles have given some idea of the reactions which take place in the early stages of activity, but further work will have to be done to confirm and extend them. The protein phosphorus, ADP, the diphosphate esters and unknown *A* have not been found to show any significant trends. ATP, orthophosphate and unknown *B* behave erratically at the beginning of activity. After 5 sec. stimulation ATP shows no change and orthophosphate and unknown *B* show a tendency to increase. The monophosphate ester fraction increases from the beginning of activity and creatine phosphate and the 'high-energy' phosphate fraction decrease. The decreases in the 'high-energy' phosphates, usually in the creatine phosphate component, at the very beginning of activity support the hypothesis that the breakdown of these compounds provides energy for mechanical work and for heat production, which, as Hill (1950*a*) has shown, starts before the mechanical changes.

The amount of 'high-energy' phosphate which has been found to break down during the first 5 sec. of activity in tortoise muscles at 0° seems to be about 40 μg. phosphorus/g. Hill (1950*a*) has shown that the heat and work produced during this period is equivalent to about 2 mcal./g. which correspond to only about 5 μg. phosphorus/g. of 'high-energy' phosphate if this is assumed to break down hydrolytically. A similar, though smaller discrepancy, was noted by Mommaerts & Rupp (1951) between the amount of ATP broken down in their experiments and that expected from the energy production. Three possible explanations, which must be tested experimentally, can be put forward in the light of the present work to account for this discrepancy. These are: (1) that the phosphate esters do not change in the early stages of activity, the changes observed being entirely due to the difference in the rates of freezing and not to the stimulation; (2) that the 'high-energy' phosphates break down hydrolytically in the early stages and that much of the orthophosphate formed combines with glycogen to form hexose monophosphate, the discrepancy in the extent of the changes being accounted for, either by the difference in the rates of freezing, or by assuming that the suddenly frozen stimulated muscle takes some time to freeze completely and is in the active state for considerably longer than a few seconds; (3) that the change which provides energy for the production of heat and work is not necessarily the hydrolytic transfer of the phosphate of the 'high-energy' fraction to water, but may be a transfer to some acceptor with which it forms either a monophosphate ester or, in the case of muscles poisoned with monoiodoacetate, a diphosphate ester, the energy released being less than in a hydrolytic splitting.

SUMMARY

1. The method of Hanes & Isherwood (1949) for separating phosphate esters by paper chromatography has been used quantitatively in the study of these esters in muscle.

2. Tests with known mixtures of phosphate esters and the recovery of the phosphorus of muscle extracts from the chromatograms suggest that the individual values obtained with the method in its present form are reliable to within 10%.

3. Trichloroacetic acid extracts of muscle have been found to contain certain phosphate esters not yet identified.

4. The pyrophosphate fraction present in the extracts is found to be entirely in the form of adenosine triphosphate and adenosine diphosphate.

5. Striated muscle from tortoise, which is about ten times slower than that from frog, contains less adenosine triphosphate and creatine phosphate + orthophosphate than the latter.

6. The chromatographic method has been used to repeat some of the classical experiments on the metabolism of intact muscle and the results of these experiments have been confirmed.

7. An attempt has been made to follow the changes in tortoise muscle during the early stages of activity. The most prominent ones are a decrease in the 'high-energy' phosphate (two-thirds adenosine triphosphate + one-half adenosine diphosphate + creatine phosphate), largely in the creatine phosphate component, and an increase in the monophosphate ester fraction.

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The Turbidimetric Estimation of Hyaluronidase

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Both the formation of turbidity by the reaction between hyaluronate and protein and the kinetics of the enzymic depolymerization of hyaluronate are complex. Hence, the turbidity-reducing action of hyaluronidase cannot be interpreted by the existing theory of enzyme kinetics. However, the use of turbidity reduction as the basis of a procedure for the assay of hyaluronidase requires that the kinetics of the reaction and the effects of the concentration and mode of preparation of both the substrate and the enzyme upon the apparent enzymic activity be known. In view of the incomplete and contradictory information available in the literature, these factors were investigated.

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MATERIALS

Crude hyaluronidase was prepared by the Freeman, Anderson, Oberg & Dorfman (1949) modification of the techniques of Madinaveitia (1939) and Hahn (1943). Fresh bovine testes were extracted with dilute acetic acid, and the extract was fractionated with ammonium sulphate, dialysed and freeze-dried. The resulting product had 28 turbidity reducing units/mg. (Meyer, 1947). In one experiment, a purified preparation which had 200 turbidity reducing units/mg. was employed. This was kindly supplied by

Dr J. Seifter of the Wyeth Institute of Applied Biochemistry, Philadelphia, Pa., U.S.A.

The preparations of hyaluronate are described elsewhere (Pearce, 1953).

METHODS

The medium used for conducting the enzymic hydrolysis was 0·1 M-acetate buffer, pH 6·0, containing 0·15 M-NaCl. In order to start the hydrolysis, equal volumes of ice-cold solutions of the enzyme and the substrate were mixed rapidly and the tube was placed immediately in a water bath kept at 37·0±0·1°. The solutions were mixed cold because of the report of McCullagh, Cassidy, Valentine & Tolksdorf (1949) that purified hyaluronidase is unstable at higher temperatures. After a period of incubation, timed to within 2 sec., the tube was transferred to a water bath kept at 70±2° for at least 30 min. in order to inactivate the enzyme. Although many workers have denatured the enzyme by heating at 60° for 10 min., the higher temperature and longer period of heating were found to be necessary in order to complete the inactivation.

After the tubes had cooled to room temperature, the hyaluronate was estimated by a technique adapted from Dorfman & Ott (1948). To 1 ml. of the buffered saline, containing hyaluronate, was added 5 ml. of an acidified protein reagent consisting of 0·1 % (w/v) of bovine plasma fraction V (albumin, Armour Laboratories) in 0·1 M-acetate buffer, pH 4·2, adjusted to pH 3·75 by the addition of 4 N-HCl. Five min. after mixing, the optical density was read in a Klett-Summerson photoelectric colorimeter using the green filter (transmission maximum at 540 mμ.). All analyses were made in duplicate.

With each experiment, a standard curve was prepared by the analysis of solutions of hyaluronate of ten different concentrations, in duplicate. The data were plotted, the points being joined by a smooth curve and the concentration of hyaluronate in the unknown tubes estimated from the graph.

RESULTS

Kinetics

The rate of destruction of the hyaluronate was determined by incubating solutions containing 100 and 500 μg./ml. with 10, 20, 50, 100, 200 and 500 μg. of hyaluronidase. At intervals ranging from 5 to 120 min. after mixing, duplicate 1 ml. samples were withdrawn, the enzyme was inactivated and the residual hyaluronate was estimated. The observed turbidities were corrected for (i) the increase in turbidity associated with the denaturation of the hyaluronidase, and (ii) the decrease in turbidity due to the slight hyaluronidase action, both occurring during the heating at 70°. The results of this experiment are presented in Fig. 1. The reactions were neither of the zero nor of the first order with respect to the apparent concentration of hyaluronate. The rate of the reaction diminished in such a manner that the destruction approached asymptotically a non-zero value that depended upon both the concentration of the enzyme and of the substrate. The possibility that the enzyme was unstable at 37° was eliminated by incubating a solution for periods of 5–120 min. at this temperature and noting its unimpaired ability to destroy hyaluronate (Table 1).

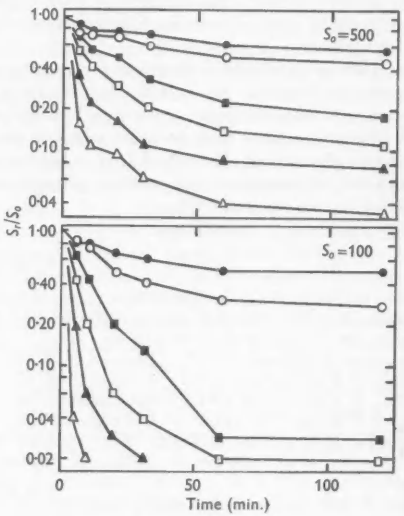

Fig. 1. The kinetics of the enzymic depolymerization of hyaluronate with 10 (●), 20 (○), 50 (■), 100 (□), 200 (▲) and 500 (△) μg. of hyaluronidase/ml. (S_o = initial concentration of hyaluronate, S_r = amount remaining after incubation, both in μg./ml.)

The kinetics during the initial stages of the reaction were investigated under conditions identical with those above, except that the times of incubation ranged from 5 to 20 min. Kinetics of the first order were followed for the first 10 min., provided that the concentration of the enzyme was less than 200 μg./ml. (Fig. 2). The extrapolation of the curves to unity at zero time shows that the mixing of the solutions at 0° did not have a significant effect on the rate of the reaction.

Table 1. *Stability of hyaluronidase at* 37°

Time of incubation (min.)	Hyaluronate destroyed (μg.)
0	270
5	280
10	280
20	280
30	270
60	270
120	265

Substrate concentration and reaction rate

The kinetics of the reaction were studied further by an examination of the effect of the concentration of the substrate upon the amount of hyaluronate destroyed during the initial first-order phase of the reaction. Solutions containing from 20 to 200 μg. of hyaluronate/ml. were incubated for 10 min. with 10, 20 and 40 μg. of hyaluronidase. After the inactivation of the enzyme, the residual hyaluronate was estimated. The results of two such experiments with the hyaluronate prepared by procedure (*a*) (Pearce, 1953) are shown in Fig. 3.

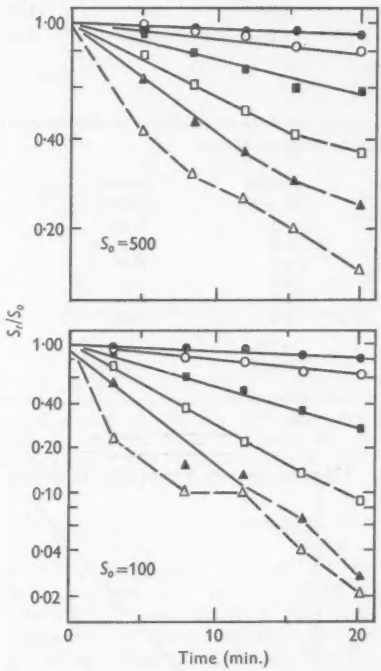

Fig. 2. The initial kinetics of depolymerization for the following concentrations of enzyme: 10 (●), 20 (○), 50 (■), 100 (□), 200 (▲) and 500 (△) μg./ml. (S_r = hyaluronate remaining and S_o = initial hyaluronate concentration, both in μg./ml.) The solid lines represent the regression equation, and the broken lines the experimental points where the relation is not linear.

Analysis of the data by plotting the reciprocal of substrate destroyed against the ratio (substrate destroyed)/(initial substrate concentration) (Lineweaver & Burk, 1934) did not give a linear plot. Hence the conventional kinetic theory was assumed not to apply.

However, a plot of the amount of hyaluronate destroyed (S_d, μg./ml.) against its initial concentration (S_o, μg./ml.) on logarithmic co-ordinates, gave a straight line, indicating that the two variables were related by a power function of the form $S_d = mS_o^b$. The values found for the exponent, b, were independent of the enzyme concentration but varied with different preparations of hyaluronate ($P < 0{\cdot}05$). The mean values for the exponent in each experiment are given in Table 2. A comparison is made in Table 3 between the values for the exponent and the measured properties of the preparations of hyaluronate (Pearce, 1953). A correlation between the exponent and the specific viscosity of the preparations was evident.

The values found for the coefficient, m, were related linearly to the concentration of the enzyme (E, μg./ml.). The values of E corresponding to $m = 0$ were calculated (column labelled e in Table 2). These could not be related to the properties of the preparation of hyaluronate and did not differ significantly from zero for any individual experiment, although the mean value did so ($P < 0{\cdot}001$). Thus the expression $a(E-e)$ may be substituted for m in the equation above.

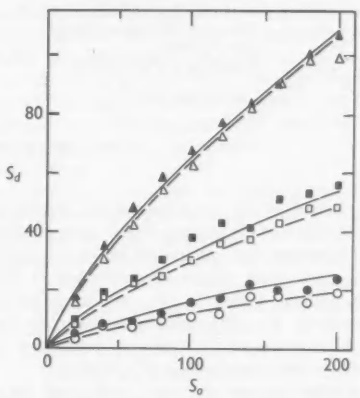

Fig. 3. The amount of hyaluronate destroyed (S_d) in the presence of various initial concentrations of hyaluronate (S_o) and 10 (● or ○), 20 (■ or □) and 40 (▲ or △) μg. of hyaluronidase/ml. The solid and open symbols represent two independent experiments. The solid lines are a plot of $S_d = 0{\cdot}056(E - 0{\cdot}46)\, S_o^{0{\cdot}73}$ and the broken lines of $S_d = 0{\cdot}059(E - 3{\cdot}15)\, S_o^{0{\cdot}73}$. All concentrations are expressed in μg./ml.

The good fit of experimental observations to the equation $S_d = a(E-e)\, S_o^b$ is indicated in Table 2. The standard errors of estimate of the substrate destroyed range from 1·7 to 4·5 μg./ml. for values ranging up to 122 μg./ml. The fit to the equation of the data obtained with hyaluronate prepared by procedure (*a*) (Pearce, 1953) is illustrated in Fig 3.

Table 2. *Constants for the equation* $S_d = a(E-e)\,S_o^b$ *with various preparations of hyaluronate*

(S_d = amount of hyaluronate destroyed. S_o = initial concentration of hyaluronate. E = concentration of enzyme, all in μg./ml. a, b and e are constants. See Pearce (1953) for the methods of preparation.)

Technique of preparation and experimental number		b	e (μg./ml.)	$\hat{S}_d$* (μg./ml.)	S.E. estimate (μg./ml.)
Digestion technique	I	0·712	0·46	38·17	1·90
	II	—	3·15	35·27	1·92
McClean, Rogers & Williams (1943)	I	0·915	4·33	18·25	3·43
	II	—	3·49	18·39	4·46
Harris & Harris (1949)	I	0·607	3·63	21·63	2·48
	II	—	3·16	28·55	2·38
Alburn & Williams (1950)		0·789	5·90	35·89	1·73
Meyer (1948)		0·639	4·58	36·35	2·02
Hadidian & Pirie (1948)		0·819	5·38	18·97	2·06

* $\hat{S}_d = a(\bar{E}-e)\,S_o^b$.

Table 3. *The properties of the various preparations of hyaluronate and the correlations of these properties with the response of the preparations to enzymic action*

Technique of preparation	Purity (%, w/w)	Protein (%, w/w)	Specific viscosity
Digestion	60	3·3	0·16
McClean *et al.* (1943)	39	17·7	0·90
Harris & Harris (1949)	57	6·1	0·26
Alburn & Williams (1950)	39	3·4	1·17
Meyer (1948)	58	2·4	0·14
Hadidian & Pirie (1948)	59	8·1	0·96
Correlation with b*	−0·651	0·746	0·812†
S_d*	0·147	−0·829	−0·409

* For meaning, see Table 2. † $P < 0{\cdot}05$.

By the use of the mean coefficient, exponent and concentration of the substrate, the mean destruction of hyaluronate was calculated for each experiment and the resulting values are recorded in Table 2. These values differed for the preparations of hyaluronate ($P < 0{\cdot}01$) and, as revealed in Table 3, they were correlated negatively with the protein content of the hyaluronate ($P < 0{\cdot}05$).

A similar experiment was performed using the purified testicular enzyme in concentrations of 2, 4 and 8 μg./ml. with the substrate prepared by the digestion procedure. The data were found to fit the equation $S_d = 0{\cdot}373(E-0{\cdot}80)\,S_o^{0{\cdot}649}$ with a standard error of estimate of 2·62 μg./ml.

The effect of the concentration of the enzyme was investigated more thoroughly by incubating 50, 100 and 200 μg. of the hyaluronate in one ml. portions of the buffered saline, for 10 min., with 5–50 μg. of hyaluronidase. The residual hyaluronate was determined in the usual manner and the data are compared in Fig. 4 with a plot of the equation

$$S_d = 0{\cdot}0455(E-0{\cdot}28)\,S_o^{0{\cdot}736}.$$

The standard error of estimate was 2·07 μg. hyaluronate/ml.

Fig. 4. The destruction of hyaluronate (S_d) with various concentrations of hyaluronidase (E) and initial concentrations of 50 (●), 100 (■) and 200 (▲) μg. of hyaluronate/ml. The lines are plotted from the equation $S_d = 0{\cdot}0455(E-0{\cdot}28)\,S_o^{0{\cdot}736}$. All concentrations are given in μg./ml.

Maximal rate of enzymic hydrolysis

The reaction was followed at concentrations of hyaluronate up to 2000 μg./ml. by reducing the volume of solution to be analysed to 0·4 ml., still using 5 ml. of the reagent of Dorfman & Ott (1948) to develop turbidity. The accuracy of these measurements was low, because the differences of turbidity were low relative to the values measured. The order of the reaction, therefore, could not be determined nor could a maximal rate be shown to have been attained. However, with concentrations of hyaluronidase up to 500 μg./ml., there were no detectable differences of rate using 1000 and 2000 μg. hyaluronate/ml. (Fig. 5), suggesting that the rate under these conditions was maximal.

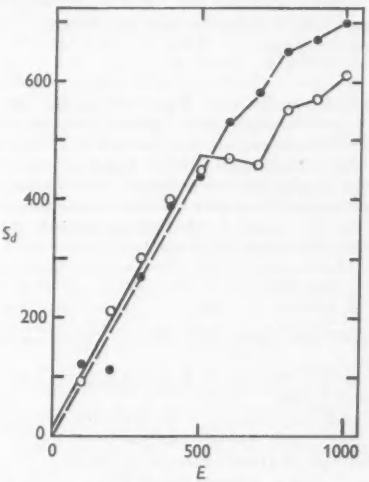

Fig. 5. The destruction of hyaluronate (S_d) by various concentrations of hyaluronidase (E) with initial concentrations of 1000 (○—○) and 2000 (●– – –●) μg. of hyaluronate/ml. With E less than 500 μg./ml., the solid lines are a plot of $S_d = 0{\cdot}93E + 9$ and the broken lines of $S_d = 0{\cdot}90E - 4$, which are the lines of best fit.

DISCUSSION

The present kinetic studies revealed that the hydrolysis of hyaluronate by hyaluronidase did not proceed according to the initial first-order kinetics for the period of 30 min. usually employed in the current procedures of assay. However, the initial reaction rate was maintained for 10 min., provided that the concentration of the enzyme was limited to approximately six turbidity reducing units (Meyer, 1947)/ml.

While the depolymerization of hyaluronate can be made to proceed at a maximal rate by the elevation of the concentration of hyaluronate to 1000 μg./ml., the small fraction of the total hyaluronate destroyed under such conditions results in a highly unreliable estimate of the enzymic activity. Hence, these conditions are worthless for assay purposes.

A superficial examination of the destruction of the substrate in relation to its initial concentration, for the range from 20 to 200 μg./ml., suggested that the reaction was proceeding at a rate intermediate between zero and first order. However, rate studies revealed that kinetics of the first order were obeyed under these conditions and, moreover, the results could not be fitted to the formulations of Lineweaver & Burk (1934). This departure from the classical kinetics was probably the result of the polymeric nature of the substrate.

A simple power function provided an excellent correspondence with the observations. This empirical relationship has proved useful in comparing a variety of preparations of the substrate. The value for the exponent was found to be correlated with the specific viscosity of the preparation which, in turn, is inversely related to the extent to which the molecule has been degraded (Ogston & Stanier, 1950; Hadidian & Pirie, 1948).

The destruction of hyaluronate was shown to be linearly related to the amount of enzyme present, but with an apparent inactivation of a small portion of the enzyme. This feature might be attributable to the production of turbidity-producing particles in increasing number during the initial phase of the reaction, as described by Alburn & Whitely (1951).

The various preparations of hyaluronate showed marked differences in their susceptibility to hyaluronidase action and the extent of the enzymic reaction seemed to be inversely related to the protein content of the hyaluronate. This observation corresponds to the finding of Kass & Seastone (1944) that mucoprotein is more resistant to enzymic action than is a purified polysaccharide. In the past, no effort has been made to control this property of the substrate, although several investigators have indicated that pure preparations of hyaluronate are required without giving any clear-cut supporting data.

Since the various factors in the equation, $S_d = a(E - e)\, S_o^b$, differ for different preparations of the substrate, this equation cannot be made the basis of the estimation of hyaluronidase until preparations of uniform properties are available. Furthermore, the activities of hyaluronidase as reported from different laboratories cannot be expected to be comparable on either an absolute or a relative basis, unless both a standard enzyme and a standard substrate have been employed.

The relationships observed between the properties of the different preparations of hyaluronate and

their behaviour as substrates may be fortuitous because the number of samples was not large and their small amounts did not permit their thorough analysis. The kinetic treatment is empirical. No simple theoretical interpretation is possible, first, because the polymeric substrate is likely to be susceptible to enzymic attack at a large number of sites and, secondly because the effect of the products of this reaction upon the formation of turbidity is not known. However, under suitable conditions, useful comparisons of enzymic activities can be made.

SUMMARY

1. The turbidity-reducing activity of hyaluronidase follows first-order kinetics under standard conditions of assay, provided that the period of incubation is limited to 10 min. and the enzyme concentration to six turbidity-reducing units (Meyer, 1947).

2. Under the above conditions, the following relationship holds: $S_d = a(E-e)\,S_o^b$, wherein S_d = the amount of substrate destroyed, S_o = the initial concentration of the substrate, and E = the concentration of the enzyme, a, b and e are constants, the first two dependent principally upon the protein content and specific viscosity of the substrate, respectively.

3. While maximal hyaluronidase activity can be attained, the conditions are not feasible for the purpose of assay.

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The Turbidimetric Estimation of Hyaluronate

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The most convenient of the several techniques that have been devised for the assay of hyaluronidase is the turbidity-reduction technique of Kass & Seastone (1944), which has been modified in various respects by several investigators (Leonard, Perlman & Kurzrok, 1946; Meyer, 1947; Warren, Durso & Levin, 1948; Dorfman & Ott, 1948; Tolksdorf, McCready, McCullagh & Schwenk, 1949; Caselli & Cutinelli, 1949; Harris & Harris, 1949; Escaron & Delfino, 1950; Alburn & Whitely, 1951).

Despite the widespread application of this procedure, the reactions upon which the assay is based have been given little critical attention. Difficulties were encountered when an attempt was made to measure hyaluronate and hyaluronidase in cultures of vaccinia virus by turbidimetric means. The formation of turbidity by the reaction of hyaluronate with protein was therefore investigated, as well as the effect of the enzyme, testicular

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hyaluronidase, upon this reaction. The former aspect of this investigation is described here.

MATERIALS

In order to acquire hyaluronate with as wide a range of properties as possible, it was prepared from human umbilical cords by several procedures:

(*a*) *Digestion technique.* The following procedure was devised following a suggestion of Hadidian (1950). Acetone-dried umbilical cords were taken up in water and digested with pepsin and trypsin. The crude hyaluronate was precipitated with ethanol, dissolved, freed of protein by treatment with $CHCl_3$: amyl alcohol and zinc hydroxide, then re-precipitated with ethanol, washed and dried.

(*b*) *Alburn & Williams* (1950). Acetone-dried cords were taken up in water and digested with pepsin and trypsin. The digest was fractionated with $(NH_4)_2SO_4$ and pyridine and the hyaluronate precipitated by means of ethanol saturated with potassium acetate.

(*c*) *Hadidian & Pirie* (1948). Acetone-dried cords were extracted with saline solution following the removal of the soluble proteins by aqueous extraction. The saline extract was fractionated with $(NH_4)_2SO_4$ and pyridine and the hyaluronate fractions were purified with $CHCl_3$: amyl alcohol and freeze-dried following dialysis.

(*d*) *Meyer* (1948). Deep-frozen umbilical cords were thawed, extracted with water and the hyaluronate of the extract precipitated as a mucin clot. The clot was dissolved and freed of protein by treatment with $CHCl_3$: amyl alcohol and zinc hydroxide. The purified hyaluronate was precipitated with ethanol.

(*e*) *McClean, Rogers & Williams* (1943). Acetone-dried cords were extracted with water and the extract, after adjustment to pH 9·5, was treated with ethanol saturated with potassium acetate to precipitate the hyaluronate.

(*f*) *Harris & Harris* (1949). The crude product, obtained by the technique of McClean *et al.* (1943), was extracted with hot water. The extract was clarified and re-precipitated at pH 9·5 with ethanol saturated with potassium acetate.

Analyses

The hexosamine content of each of the preparations of hyaluronate was determined as follows. The polysaccharide was hydrolysed according to the conditions of Palmer, Smyth & Meyer (1937). The hydrolysate was dried, as suggested by Hadidian & Pirie (1948) and the amino sugar was estimated by a procedure modified from that of Blix (1948). A correction was made for the hexosamine destroyed during the hydrolysis and, from the corrected values, the purity of the preparation of hyaluronate was calculated, assuming pure hyaluronate to contain equimolar amounts of glucuronic acid and *N*-acetylglucosamine combined through glycosidic linkages. The nitrogen was estimated by the micro-Kjeldahl nesslerization procedure of Koch & McMeekin (1924) and the protein content calculated from the non-glucosamine nitrogen, assuming that the protein contains 16% N. Specific viscosities were determined according to the conditions of Hadidian & Pirie (1948), with a preliminary adjustment of the concentrations of hyaluronate according to the results of the hexosamine analyses.

EXPERIMENTAL AND RESULTS

The effect of various preparations of hyaluronate upon turbidity

The following modification of the procedure of Dorfman & Ott (1948) was employed: to 1 ml. of 0·1 M-acetate buffer, pH 6·0, containing 0·15 M-NaCl and 20–200 μg. of hyaluronate, was added 5 ml. of a reagent consisting of 0·1% (w/v) of albumin (bovine plasma fraction V, Armour Laboratories) in 0·1 M-acetate buffer, pH 4·2, adjusted to pH 3·75 by the addition of 4 N-HCl. Five min. ($\pm$15 sec.) after the addition of the reagent, the optical density was read in a Klett-Summerson photoelectric colorimeter using the green filter (maximal transmission at 540 mμ.).

The turbidity increased with the concentration of the hyaluronate, but neither in direct proportion, as found by Dorfman & Ott (1948), nor according to the sigmoidal relationship reported by Meyer (1947) and others. Rather, the relationship was described by a power function over the range 20–200 μg. of hyaluronate/ml. As an example, the experimental findings obtained with the hyaluronate prepared by the digestion technique (*a*, above) fitted closely the equation $T = 2{\cdot}25S^{0{\cdot}725}$, as shown in Fig. 1. ($T$ = colorimeter scale reading $\simeq 500 \times$ optical density; S = concentration of hyaluronate, in μg./ml.)

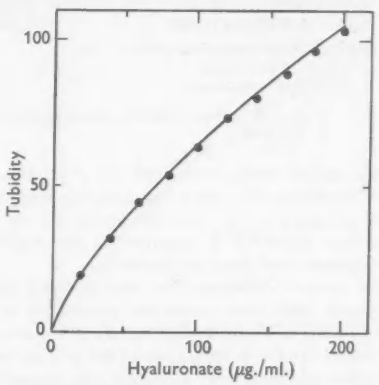

Fig. 1. The turbidity (T) with various concentrations of hyaluronate (S). The experimental points are compared with a plot of $T = 2{\cdot}25S^{0{\cdot}75}$.

The other preparations also yielded data that fitted well a power function, as evidenced by the standard error of estimate of turbidity, which ranged from 0·88 to 3·69 for turbidity values from 10 to 110 (see Table 1). This accuracy was as good as was expected since the colorimeter was read to the nearest unit of the scale.

The values observed for the exponent and coefficient with the various preparations of hyaluronate are recorded in Table 1. The values of the exponent did not differ for the various preparations

Table 1. *The constants of the equation, $T = aS^b$, relating turbidity (T) to the concentration of hyaluronate (S)*

Preparation and expt. no.		b	a*	S.E. of estimate of turbidity*
Digestion technique	I	0·762	1·921	1·39
	II	0·711	1·898	1·00
	III	0·730	1·899	2·46
	IV	0·764	1·806	1·49
	V	0·723	1·815	2·18
Meyer (1948)	I	0·746	1·825	1·58
	II	0·721	1·833	1·43
McClean *et al.* (1943)	I	0·748	1·864	0·88
	II	0·828	1·834	1·44
Harris & Harris (1949)	I	0·731	2·006	1·76
	II	0·747	2·066	1·75
Hadidian & Pirie (1948)		0·760	1·898	3·69
Alburn & Williams (1950)		0·836	1·255	1·17

* The coefficients and S.E.'s of estimate were calculated using a mean value, 0·754, for the exponent b.

Table 2. *The relation of the coefficient to the properties of the hyaluronate*

Preparation	Coefficient: Observed	Coefficient: Corrected*	Specific viscosity	Purity (%, w/w)	Protein (%, w/w)
Digestion technique	1·868	2·747	0·16	60	3·3
Harris & Harris (1949)	2·036	3·132	0·26	57	6·1
Hadidian & Pirie (1948)	1·898	2·833	0·96	59	8·1
McClean *et al.* (1943)	1·849	3·773	0·90	39	17·7
Meyer (1948)	1·829	2·771	0·14	58	2·4
Alburn & Williams (1950)	1·255	2·561	1·17	39	3·4
Coefficient of correlation with					
Observed coefficient			−0·612	0·656	0·255
Corrected* coefficient			0·074	—	0·908†

* $a' = a/P^b$ where a and a' are observed and corrected coefficients. P is the purity of the hyaluronate.
† $P < 0·05$.

but the values of the coefficient did ($P < 0·01$). The mean coefficient for each preparation was calculated, using the mean exponent for all the preparations and provided a measure of the turbidity development with each preparation.

The mean coefficient for each preparation is compared with the measured properties of the hyaluronate in Table 2. No significant correlation was found to exist between any of the properties and the observed coefficient. However, the preparations differed appreciably in purity as indicated by their differing contents of hexosamine. Therefore, the coefficient for each preparation was corrected for the lack of purity by the expression $a' = a/P^b$, which was derived from the series of equalities

$$T = aS^b = (a/P^b)\ (PS)^b = a'S'^b,$$

in which S' = the corrected concentration of hyaluronate, a and a' = the observed and corrected coefficients respectively, b = the exponent and P = the purity of the preparation, calculated from its hexosamine content and expressed as a decimal fraction. The values of the corrected coefficients were slightly correlated with the protein contents of the preparations ($P < 0·05$).

Kinetics

One ml. portions of 0·15 M-NaCl, buffered with 0·1 M-acetate at pH 6·0 and containing 100, 250, 750 or 1000 μg. of hyaluronate, were treated with 5 ml. of the albumin reagent of Dorfman & Ott (1948). The turbidity at 540 mμ. was measured as soon as possible thereafter and at intervals up to 100 min. The hyaluronate used in this and subsequent experiments was prepared by the digestion technique (*a*, above).

The turbidity rose rapidly to a maximum and then declined gradually, as shown in Fig. 2. The law of mass action evidently applied to the initial reaction, since the rate of formation of turbidity was greater with 250 than with 100 μg. and was too rapid to follow with higher concentrations.

Effect of pH

Solutions of 0·5 M-sodium acetate and 0·5 M-acetic acid, both containing 0·5% (w/v) of albumin, were mixed in various proportions to give pH values from 3·0 to 5·0. To 1 ml. portions of each of these buffers, 4 ml. of 0·1% (w/v) of hyaluronate were added and the turbidity was measured as described in the first paragraph of the Experimental section. The resultant pH in each tube was checked electrometrically using a glass electrode.

An appreciable turbidity developed only between pH 3·8 and 4·4 with the high values close to 4·2, as shown by Fig. 3. The turbidity was negligible above pH 4·6 and below 3·5.

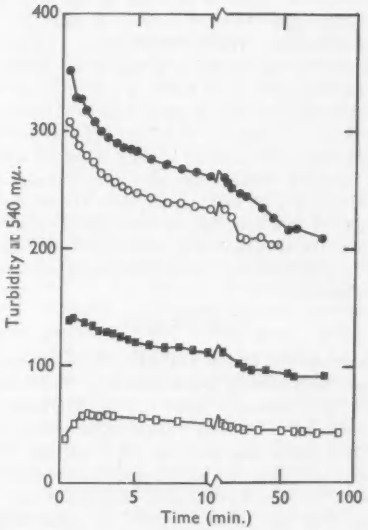

Fig. 2. The kinetics of turbidity formation for 100 (□), 250 (■), 750 (○) and 1000 (●) μg. of hyaluronate. The scale is compressed tenfold after 10 min.

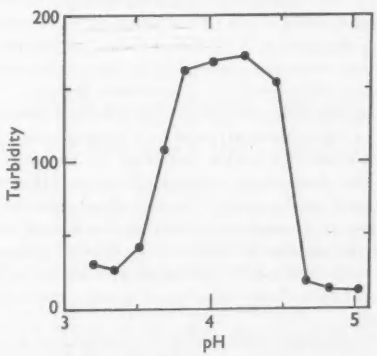

Fig. 3. The effect of pH upon turbidity development.

Effect of ionic strength

Acetate. M-Acetate buffer, pH 4·2, and water, both containing 0·5% (w/v) of albumin, were mixed to give concentrations of acetate from 0·1 to 1·0M. A solution of hyaluronate was added to 1 ml. portions of each solution and the turbidity was measured as already described.

The results, presented in Fig. 4, revealed that the elevation of the concentration of acetate interfered with the development of turbidity.

Sodium chloride. To 1 ml. portions of 0·1M-acetate buffer, pH 4·2, containing 0·5% (w/v) of albumin and from 0·1 to 1·0M-NaCl, hyaluronate was added and the turbidity measured.

Sodium chloride was much more effective than acetate in inhibiting the development of turbidity and, with concentrations of sodium chloride above 0·5*N*, the turbidity fell to low values (see Fig. 4).

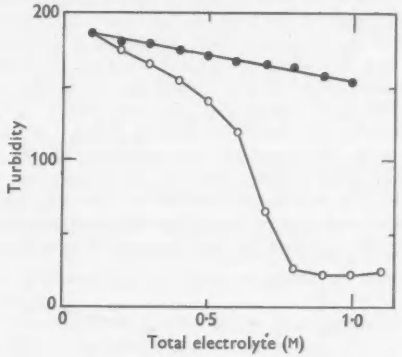

Fig. 4. The effect of the concentration of acetate buffer, pH 4·2, (●) and NaCl (○) upon the development of turbidity.

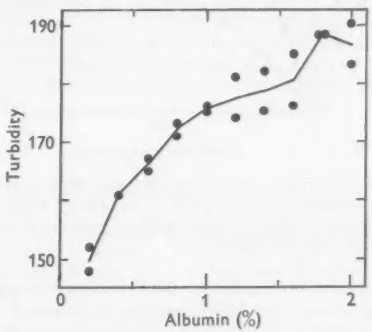

Fig. 5. The effect of the concentration of albumin upon the development of turbidity. The mean values are joined by the solid line, but the individual observations are plotted to illustrate the increasing irregularities at the high concentrations.

Effect of concentration of albumin

A 0·5M-acetate buffer, pH 4·2, and 2% (w/v) solution of albumin in the buffer were mixed to give concentrations of albumin from 0·2 to 2·0% (w/v). One ml. portions of these solutions were treated with hyaluronate and the turbidity measured.

The turbidity increased as the concentration of albumin was elevated but the values became irregular with high concentrations due to the formation of visible clot-like aggregates (Fig. 5).

Recommended procedure

These experiments suggested two methods for increasing the sensitivity of the estimation of hyaluronate. First, the total concentration of electrolytes could be reduced by using acetic acid in the protein reagent to bring the pH to 4·2, rather than acetate buffer. Secondly, the relative proportions of the protein reagent and of the solution of hyaluronate in the final mixture might be reversed.

The application of these principles is illustrated by the following procedure: to 4 ml. of 0·05 M-acetate buffer, pH 6·0, containing 0·05 M-NaCl and 20–200 μg. of hyaluronate/ml., was added 1 ml. of 0·568 N-acetic acid, containing 0·5 % (w/v) of albumin. The turbidity was measured at 540 mμ. 5 min. after mixing. The pH of the mixture was 4·20. The electrolyte content of the solution of hyaluronate was reduced from that used previously in order to permit the development of turbidity.

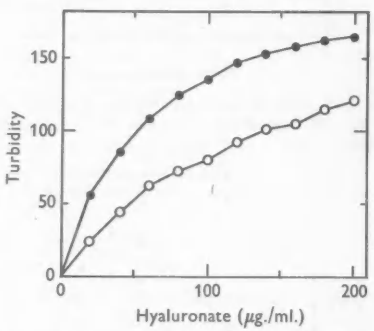

Fig. 6. Standard curves for the new procedures applied to 0·05 M-acetate buffer, pH 6·0, containing 0·05 M-NaCl (●) and to virus culture medium, buffered to pH 6·0 with 0·0791 M-NaH_2PO_4 (○).

A plot of the turbidity against the concentration of hyaluronate yielded a smooth uninflected curve which, however, did not fit a power function (Fig. 6). The four lower points corresponded to the equation $T = 9{\cdot}47S^{0{\cdot}607}$ with a standard error of estimation of 3·1 units of the colorimeter scale. The fourfold increase in sensitivity was relative rather than absolute, since four times the weight of hyaluronate was required. A procedure of this sort has value only if relatively large volumes of a dilute solution of hyaluronate, low in electrolytes, are to be analysed.

A solution of hyaluronate in 0·1 M-acetate buffer, pH 6·0, containing 0·15 M-NaCl could be analysed by treating a 1 ml. portion with 5 ml. of 0·0491 N-acetic acid, containing 0·1 % (w/v) of albumin. This solution yielded a final pH of 4·22. The data fitted a simple power function of the concentration of hyaluronate, $T = 2{\cdot}92S^{0{\cdot}703}$, with a standard error of estimate of 1·37. This procedure was only 8 % more sensitive than the usual technique, but the reagent was simpler in composition.

A slight modification of the procedure recommended above could be applied to the analysis of hyaluronate dissolved in the virus culture medium of Simms & Sanders (1942). The medium was buffered to pH 6·0 by the addition of an equal volume of 0·0791 M-NaH_2PO_4 and 4 ml. of the mixture were treated with 1 ml. of 0·179 N-acetic acid containing 0·5 % (w/v) of albumin. The final pH was 4·10.

A smooth curve, similar to that obtained with the dilute acetate buffer, was also found under these conditions (Fig. 6) and, again, only concentrations of 60 μg. of hyaluronate, or less, fitted to a power function. In this instance, the equation of best fit was $T = 1{\cdot}87S^{0{\cdot}855}$ with a standard error of estimate of 0·21 unit.

DISCUSSION

The mechanism of the turbidity-forming reaction has not been clearly demonstrated. Most investigators have assumed that a carbohydrate anion combines with a protein cation by an electrostatic bond to form a mucoprotein. The optimal pH and the effect of electrolytes provide some additional evidence for the hypothesis. Acidification of the system has the double effect of enhancing the cationic ionization of the protein and depressing the ionization of the hyaluronate anion. Thus, the maximal ionization of both hyaluronate and protein cannot be attained simultaneously, and the observed optimal pH of the reaction represents the best compromise. The interference of electrolytes with the formation of turbidity can be understood in terms of their effect on interionic forces.

In a practical procedure, the minimal concentration of electrolyte attainable is determined by the requirement for stable buffering on the one hand and the electrolyte content of the solution to be analysed on the other. An increased sensitivity of the reaction can be attained by the use of a weak acid, rather than a buffer, in the protein reagent, so that the cations of the solution containing hyaluronate provide the other component of the buffering system.

The increased sensitivity of the reaction, achieved by increasing the concentration of albumin, is offset by the irregular values that result from the formation of grossly visible particles in the mixture. The concentration recommended appeared to be a reasonable compromise between sensitivity and reproducibility.

The kinetics of the development of turbidity confirm the reported necessity for timing the period between the addition of the albumin reagent and the measurement of the turbidity. In choosing a time interval for routine assays, the increased sensitivity

attainable by making an early reading is offset by the additional effort involved in accurate timing. However, the time cannot be shortened beyond the period required for the maximal development of turbidity. The formation of turbidity apparently follows the law of mass action as judged by the time required to reach a maximum with various concentrations of hyaluronate. The decay phase of the reaction seems to be characteristic of the system, since the values for the different preparations of hyaluronate run parallel on a logarithmic plot.

Since the development of turbidity is dependent upon the properties of the hyaluronate, this reaction should be applied with caution to the estimation of hyaluronate of unknown composition. The possible difficulties encountered in the application of this reaction to systems not under strict control is illustrated by our experience with the analysis of virus cultures. Although smooth curves and excellent precision were found upon addition of solutions of hyaluronate to the medium for virus culture, the recovery of hyaluronate from the same medium containing, in addition, growing chick-embryo mince and vaccine virus was variable and averaged only 66%. This low recovery was not attributable to any of the factors studied.

The turbidity-forming reaction between hyaluronate and protein therefore provides a satisfactory method for the estimation of the polysaccharide in controlled systems, such as are commonly employed in enzyme assays but cannot, at the present, be applied with confidence even to simple biological systems.

SUMMARY

1. The effects of pH, and of the concentrations of hyaluronate, protein and electrolyte upon the turbidity-producing reaction between hyaluronate and protein have been studied, as well as the kinetics of the reaction.

2. The turbidity produced is a simple power function of the concentration of hyaluronate and depends upon the properties of the preparation, principally its purity and content of protein.

3. The optimal pH of the reaction is 4·1–4·2.

4. Electrolytes interfere with the formation of turbidity, sodium chloride being more active in this respect than acetate.

5. The increased sensitivity achieved by raising the concentration of albumin is offset by a loss of precision.

6. The turbidity develops rapidly at a rate dependent upon the law of mass action and decays at a diminishing rate over a period of several hours.

7. The application of these observations to some practical analytical problems is illustrated.

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Aerobic Metabolism of the Hepatopancreas of *Helix pomatia*

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The presence of the tricarboxylic-acid cycle (Krebs's cycle) has been demonstrated in most mammalian tissues investigated (Krebs & Johnson, 1937; Green, Loomis & Auerbach, 1948), where it appears to be the major pathway for the oxidation of carbohydrate, fat and protein intermediaries. No detailed examinations have been made in the invertebrates. However, evidence has been presented of Krebs's cycle activity in certain nematodes (Massey & Rogers, 1950), the cockroach (Barron & Tahmisian, 1948) and *Drosophila* (Spirtes, 1951). No similar studies have been made in *Helix pomatia*. The evidence of Biedermann (1911), Baldwin (1938) and Vonk, Mighorst & de Groot (1950) suggests that the hepatopancreas plays an important role in the metabolism of the animal.

The enzyme systems examined in the present investigation were those contained in suspensions and mitochondrial preparations from the hepatopancreas of both hibernating and active *H. pomatia*, since there is evidence of changes in the enzymes of an organism during hibernation (Baldwin, 1938; Suomalainen, 1935).

METHODS

The snails used in these experiments were either kept in the refrigerator in the hibernating state, or had been active and fed on cabbage in the laboratory at 22° for at least 7 days before use. Unless otherwise specified, results reported in this paper were obtained with preparations from active *H. pomatia*.

Enzyme preparations

Suspensions. The hepatopancreas was rapidly removed and ground in an ice-cold Potter 'homogenizer' in either 0·2 M-sucrose solution or 0·5% KCl solution. The suspension was diluted with the appropriate medium to give a final concentration of approximately 20% tissue.

Mitochondria. These were isolated according to the methods described by Schneider (1948) using 10% hepatopancreas suspensions made in 0·2 M-sucrose. All preparations were carried out in a cold room at 2°, a Servall SS-1-*a* high-speed centrifuge being used for the isolation of the mitochondria.

Hexokinase. This was obtained by the method of Berger, Slein, Colowick & Cori (1946) as described by Cross, Covo, Taggart & Green (1949). The preparation was stored as a frozen solution at −10° and retained its activity for many months. The conditions for autolysis for baker's yeast were those described by Bailey & Webb (1948).

Determinations

Inorganic orthophosphate was determined by the method of Fiske & Subbarow (1925), and α-ketoglutarate and pyruvate by the method of Friedemann & Haugen (1943). Citrate was determined by the method of Natelson, Pincus & Lugovoy (1948) and sometimes by that of Weil-Malherbe & Bone (1949). Succinate was determined by the method of Krebs (1937). O_2 uptakes were determined manometrically; the gas phase was air.

Reagents

Adenosine triphosphate (ATP) was prepared by the method of Dounce, Rothstein, Beyer, Meier & Freer (1948). By chemical and spectrophotometric analysis (Kalckar, 1947) it was found to be 98% pure.

Commercial adenosine 5′-phosphate was crystallized twice from hot water. On spectrophotometric analysis it was found to be 99% or more pure.

Sodium citrate, L-glutamate, L-malate, α-ketoglutarate and oxaloacetate were commercial preparations. Succinic acid was recrystallized three times from hot water. The succinic acid was dissolved, neutralized and the sodium salt precipitated with ethanol. The salt was dissolved and crystallized from aqueous ethanol. Octanoic acid was obtained commercially and distilled *in vacuo*. Cytochrome *c* was prepared by the method of Keilin & Hartree (1937) and dialysed against distilled water. Sodium pyruvate was prepared from commercial pyruvic acid after distillation *in vacuo*, by a method described by Lardy (1949).

Diphosphopyridine nucleotide (DPN) was prepared by an unpublished method of Ochoa, and coenzyme A by the method of Lipmann, Kaplan, Novelli & Tuttle (1950). Hexose diphosphate and phosphoglyceric acid were gifts from Mr J. H. Ashby and by chemical analysis were 98% pure. Hexose monophosphate was a gift from Dr J. D. Judah.

Standard medium. Final concentrations in the Warburg flask were 0·025 M-KCl; 0·0066 M-Mg^{2+}; 0·001 M-adenylic acid (AMP); 1×10^{-5} M-cytochrome *c*; 0·014 M-sodium phosphate buffer, pH 7·4; substrate as indicated in text. Final volume was made up to 3 ml. with water. The enzyme preparations were added in either KCl or sucrose solution (0·5 ml.). 0·1 ml. 20% KOH was placed in the centre well with filter papers. The equilibration was carried on for 10 min.

RESULTS

The endogenous respiration of suspensions prepared in 0·5% KCl or 0·2 M-sucrose from both active and hibernating specimens of *H. pomatia* was measured. No significant difference was observed between the levels of the endogenous respiration of the active and hibernating snails as shown in Table 1.

Table 1. *Effect of Krebs's cycle intermediates on the respiration of hepatopancreas suspensions*

(System: standard medium, 30 μmoles substrate added except in the case of succinate where 100 μmoles added. Temp. 38°.)

Condition of animal	Substrate	O_2 uptake (μl./mg. N/hr.) Mean	Range	No. of observations
	(1) 0·5% KCl suspensions			
Active	Nil	11	9·3–14·1	5
Hibernating	Nil	12	9·1–15·5	7
Active	Succinate	36	25–43·5	8
Hibernating	Succinate	39	37–41·5	6
Active	α-Ketoglutarate	16	12·1–18·4	5
Hibernating	α-Ketoglutarate	17	10·8–23·0	5
Active	Citrate	19	16·6–19·9	6
Hibernating	Citrate	19	9·6–27·8	7
Active	Fumarate	15	11·6–16·9	5
Hibernating	Fumarate	21	14·7–24·4	6
Active	L-Malate	18	13·2–25·6	5
Hibernating	L-Malate	17	10·2–19·5	5
	(2) 0·2M-Sucrose suspensions			
Active	Nil	14	11·9–15·4	5
Hibernating	Nil	10	8·1–13·1	6

The effect of the addition of various glycolytic intermediates on the respiration of 0·2M-sucrose suspensions was next investigated (Table 2). All substrates tested were found to increase the rate of respiration. Baldwin (1938) observed that tissue slices of the hepatopancreas of active *H. pomatia* utilize galactose, whereas preparations from hibernating animals do not. It has now been demonstrated that galactose was oxidized by 0·2M-sucrose suspensions of the hepatopancreas prepared from the active animal, but not by similar preparations from hibernating animals (Table 2).

Table 2. *Effect of added carbohydrates and glycolytic intermediates on the respiration of 0·2M-sucrose suspensions of the hepatopancreas*

(System: standard medium, 30 μmoles substrates added. Tissue added equivalent to 4 mg. N. Temp. = 38°. Duration of experiment = 30 min.)

Condition of animal	Substrate added	O_2 uptake (μl.)
Active	Nil	22
	Galactose	34
	Fructose 6-phosphate	34
	Hexose diphosphate	36
	Phosphoglyceric acid	42
	Glucose	35
Hibernating	Nil	16
	Phosphoglyceric acid	32
	Galactose	17

In Table 1 are also shown the results of the addition of Krebs's cycle intermediates to 0·5% potassium chloride suspensions prepared from the hepatopancreas of both active and hibernating animals. All of the Krebs's cycle intermediates tested were found to increase the rate of respiration of the suspension. The extent of the increase due to the addition of the substrate was of the same order for the hepatopancreas preparations from both active and hibernating snails. The addition of Krebs's cycle intermediates to hepatopancreas suspensions after some 30 min. incubation in the Warburg bath was found to restore the level of the endogenous respiration, the endogenous substrate presumably being exhausted.

Table 3. *Effect of malonate on the oxidation of glucose and galactose in the presence and absence of malate*

(System: standard medium, malonate final concentration = 0·033M; glucose and galactose 30 μmoles. 0·2M-sucrose suspension added equivalent to 4 mg. N. Temp. = 38°. Duration of experiment = 30 min.)

Additions	Oxygen uptake (μl.)
Nil	38·0
Malonate	32·0
Glucose	43·0
Glucose + malonate	37·0
Glucose + malonate + 5 μmoles malate	48·0
Galactose	46·0
Galactose + malonate	37·0

The effect of malonate on the respiration of hepatopancreas preparations

The effect of malonate on the respiration of a 0·2M-sucrose suspension in the presence of glucose, galactose, and L-malate was investigated. The results in Table 3 show that the addition of malonate causes some reduction in the oxidation of glucose, but that the original level is restored by the addition

of L-malate. Malonate was also found to inhibit the oxidation of all the Krebs's cycle intermediates tested (Table 4).

Table 4. *Effect of malonate on the oxidation of Krebs's cycle intermediates by 0·5 % hepatopancreas suspensions*

(System: Standard medium, substrates 30 μmoles (succinate 100 μmoles), malonate 0·033 M. Tissue suspension added equivalent to 3 mg. N. Temp. = 38°. Duration of experiment = 1 hr.)

Substrate	O_2 uptake (μl.) No malonate	O_2 uptake (μl.) With malonate	Inhibition (%)
Nil	30·0	25·0	16·0
Succinate	362	62·0	86·0
Fumarate	167	47·0	72·0
Citrate	60	36·0	40·0
α-Ketoglutarate	40	25·0	38·0

It is considered that these experiments indicate that the hepatopancreas of *H. pomatia* contains enzymes which will oxidize Krebs's cycle intermediates. The inhibition of the endogenous respiration which occurs on the addition of malonate, and the effect of the latter on the oxidation of all added Krebs's cycle intermediates, suggests that succinoxidase and possibly the Krebs's cycle plays a part in the endogenous respiration. The individual reactions of the Krebs's cycle were next investigated.

Reactions of the Krebs's cycle

The reaction citric acid → α-ketoglutarate was examined, further oxidation of α-ketoglutarate being prevented by the addition of arsenite. In Table 5 are shown the results of some typical experiments. The addition of citric acid to the hepatopancreas suspension was found to increase considerably the amount of α-ketoglutarate which accumulated.

Table 5. *Conversion of citric acid to α-ketoglutarate in 0·5 % KCl suspension of the hepatopancreas of active* Helix pomatia

(System: standard medium and final arsenite concentration = 0·006 M. Suspension added equivalent to 4 mg. N. Duration of experiment = 1 hr.)

Additions	Oxygen utilized (μatoms)	Citrate utilized (μmoles)	α-Keto-glutarate formed (μmoles)
Nil	13·0	1·5	2·0
30 μmoles citrate	18·2	11·0	9·0
30 μmoles citrate	13·2	10·0	7·0
30 μmoles citrate	11·0	9·0	7·5
No arsenite +30 μmoles citrate	5·7	7·0	4·1

In Table 6 are shown the results of experiments demonstrating the synthesis of citrate from a series of precursors. All the reactions were investigated in Thunberg tubes under anaerobic conditions to

Table 6. *Synthesis of citrate by hepatopancreas preparations*

(System: 0·0066 M-Mg^{2+}; sodium phosphate buffer, pH 7·4, 0·014 M; ATP as indicated in the table; 20 μmoles substrates. Final volume = 3 ml. Duration of experiment = 30 min. Temp. = 28°. Results expressed as μg. citrate synthesized/mg. N of suspension or mitochondria. Tissue suspensions or mitochondria added equivalent to 4 mg. N.)

	Oxalo-acetate	HCO_3'	Pyruvate	ATP concn. ($\times 10^{-3}$ M)	Citrate synthesized (μg.)
(1) KCl suspension from active animal	+	−	+	2	13
	+	−	+	1	18
	+	−	+	2	20
	+	−	+	3	24
	−	+	+	1	19
	−	−	−	1	0
(2) Mitochondria from active animal	+	·	+	1	20
	−	+	+	1	0
(3) KCl suspension from hibernating animal	+	−	+	1	10
	−	+	+	1	0
	−	−	−	1	0

Table 7. *Accumulation of succinate by 0·5 % KCl suspensions of the hepatopancreas of active* Helix pomatia

(System: standard medium; final concentration of malonate = 0·033 M. Temp. = 38°. Duration of experiment = 60 min. Suspension added equivalent to 4 mg. N.)

Additions	Oxygen utilized (μatoms)	α-Ketoglutarate Present (μmoles)	α-Ketoglutarate Utilized (μmoles)	Succinate formed (μmoles)
Nil	3·5	1·0	—	2·0
10 μmoles α-ketoglutarate	5·0	8·5	1·5	4·0

Table 8. *Intracellular distribution of succinoxidase, cytochrome oxidase and nitrogen in 0·2 M-sucrose suspensions of hepatopancreas*

(Results expressed as percentage of original enzyme activity or nitrogen content.)

Condition of animal	Enzyme system	Whole suspension	Supernatant I	Cellular debris	Mitochondria	Supernatant II
Active	Succinoxidase	100	75	16	69	4
Active	Cytochrome oxidase	100	79	2	43	32
Active	Nitrogen	100	82	19·9	14	54·5
Hibernating	Nitrogen	100	79	17	13·5	55·0

prevent further utilization of the citrate formed. Citrate synthesis was not investigated in sucrose suspensions since sucrose interferes with the analysis for citric acid.

The reaction α-ketoglutarate → succinate was studied with the aid of malonate to prevent further oxidation of succinate. Respiring suspensions of the hepatopancreas accumulated succinate in the presence of malonate and the yield of succinate was increased by the addition of α-ketoglutarate. The results of these experiments are shown in Table 7.

The intracellular localization of Krebs's cycle enzymes in the hepatopancreas of Helix pomatia

Differential centrifugations were carried out on 0·2 M-sucrose suspensions prepared from the hepatopancreas of both active and hibernating *H. pomatia*. The tissue suspension was centrifuged at 3000 *g* for 10 min., and the supernatant (S_1) centrifuged for 10 min. at 15000 *g* at 2° to sediment the mitochondria from the supernatant (S_2). The mitochondria were washed by suspension in 0·2 M-sucrose and centrifugation at 15000 *g*. The distribution of the nitrogen, succinoxidase and cytochrome oxidase of these fractions are shown in Table 8. Succinoxidase and cytochrome oxidase were determined by the method of Schneider & Potter (1943).

Table 9. *Oxidation of Krebs's cycle intermediates and* L-*glutamate by mitochondria isolated from the hepatopancreas of active* Helix pomatia

(System: standard medium; 30 μmoles substrates (succinate 100 μmoles). Temp. = 38°.)

Substrate	Oxygen uptake (μl./mg. N./hr.)
Nil	0
α-Ketoglutarate	24·0
L-Malate	44·0
Succinate	110·0
Citrate	7·0
L-Glutamate	0

The oxidation by mitochondria of α-ketoglutarate, L-malate, fumarate, succinate and citrate was investigated and the results are shown in Table 9. Additions of DPN were found to increase the rate of oxidation of L-malate and α-ketoglutarate (Table 10).

Table 10. *Effect of DPN additions on the oxidation of* L-*malate and α-ketoglutarate by hepatopancreas mitochondria*

(System: standard medium; additions as described in the table. Mitochondria added equivalent to 3 mg. N. Temp. = 38°. Duration of experiment = 30 min.)

Additions	Oxygen uptake (μl.)
30 μmoles malate	26·0
30 μmoles malate + 1 μmole DPN	47·0
30 μmoles α-ketoglutarate	28·0
30 μmoles α-ketoglutarate + 1 μmole DPN	37·0

The synthesis of citric acid from pyruvate and oxaloacetate by mitochondrial preparations of both active and hibernating animals was investigated. It was found that the synthesis of citrate was proportional to added ATP but that the addition of coenzyme A had no effect on the reaction. The synthesis of citric acid from added bicarbonate and pyruvate took place in the presence of mitochondrial preparations from active animals but not in that of preparations of hibernating animals (Table 6).

Acetate and octanoate oxidation with hepatopancreas mitochondria and suspensions was studied using L-malate or succinate as primer. Both in the absence and presence of primer, acetate and octanoate failed to increase the oxygen uptake above that shown by the control. Substitution of ATP and/or the addition of DPN or coenzyme A failed to elicit oxidation of acetate or octanoate.

Investigations of oxidative phosphorylation in the hepatopancreas

The esterification of inorganic phosphate was studied in sucrose suspensions of the hepatopancreas. Sodium fluoride was added to inhibit adenosine triphosphatase activity. The effect of the oxidation of Krebs's cycle intermediates on the level of inorganic phosphorus esterified was tested. The increased oxygen uptake in the presence of these intermediates was found not to be associated with phosphorylations. The phosphate that was esterified during the experiment was found to be unaffected by 2:4-dinitrophenol (DNP) (Table 11).

Experiments were carried out over a range of temperatures, 22–38°, but lowering the temperature did not initiate oxidative phosphorylation associated with added Krebs's cycle intermediates. The addition of respiratory co-factors (coenzyme A, DPN, triphosphopyridine nucleotide (TPN), thiamine pyrophosphate, or flavin-adenine dinucleotide) to the hepatopancreas suspensions did not elicit oxidative phosphorylation.

Table 11. *Effect of Krebs's cycle intermediates and dinitrophenol on phosphate esterification by tissue suspensions*

(System: standard medium; 90 μmoles glucose; 0·05 ml. hexokinase solution; dinitrophenol final concentration 10^{-5} M. Tissue added equivalent to 6 mg. N. Temp. = 25°. Duration of experiment = 90 min.)

Substrate	Dinitrophenol	P esterified (μmoles)
Nil	–	13·0
Nil	+	9·0
Succinate	–	14·0
Succinate	+	13·0
L-Glutamate	–	14·0
L-Glutamate	+	14·0
L-Malate	–	13·0
L-Malate	+	13·0

In view of the experiments of Kielley & Kielley (1951) measurements were made both in the presence and absence of fluoride. With suspensions, a greater esterification was found in the presence of sodium fluoride. Numerous experiments were made with mitochondrial preparations. These were carried out with and without sodium fluoride; with added myokinase (Kalckar, 1947); with both ADP and AMP as phosphate acceptors and with and without the respiratory co-factors listed above. In no case was any phosphorylation observed during the oxidation of Krebs's cycle intermediates, which included succinate, L-malate, fumarate and α-ketoglutarate. L-Glutamate was also tested in this system, but without effect.

The absence of any esterification of phosphate by the mitochondrial preparations and the stability of the phosphate esterified in the suspensions to DNP suggest that these phosphorylations result from anaerobic reactions, e.g. glycolytic reactions. We have found in fortified rat-liver suspensions that the phosphorylations associated with glycolysis are not interrupted by DNP.

DISCUSSION

The snail, as a poikilothermic animal, has in the laboratory a body temperature of about 20–23°. Manometric measurements of the hepatopancreas at this temperature were so small that for the determination of μl. O_2/mg. N/hr., a working temperature of 38° was adopted. No instability in the respiration of preparations in the presence of substrates was noted during experiments of 40 min. duration.

The activity shown by the hepatopancreas suspensions is about one-tenth that of similar preparations of rat liver. From a study of the data of Massey & Rogers (1950) it would appear that the nematode tissue examined possessed an aerobic respiration of the same order as that of the hepatopancreas. These activities are low in contrast to those obtained by us for muscle preparations of *Locusta migratoria* (unpublished) and for cockroach muscle (Barron & Tahmisian, 1948).

It was not possible in *H. pomatia* to investigate muscle. The consistency of the foot muscle is such that it did not permit the preparation of tissue dispersions which could be examined, while the columella muscle in this species is so small that it is not practicable for investigations.

The evidence for the presence of Krebs's cycle activity in the hepatopancreas may be summarized as follows:

(1) The addition of fumarate, α-ketoglutarate, citrate, *cis*-aconitate, succinate, malate and oxaloacetate caused an increase in the oxygen uptake.

(2) In the presence of malonate, succinate accumulated and the amount formed was increased by the addition of α-ketoglutarate.

(3) Malonate inhibited the respiration, and this inhibition was partially removed when intermediates of the Krebs's cycle were added.

(4) Added citrate was utilized by the suspension and α-ketoglutarate accumulated in the presence of arsenite. Even in the absence of inhibitor, however, α-ketoglutarate accumulated during the oxidation of citrate.

(5) Small quantities of citrate were synthesized by the hepatopancreas preparations from added pyruvate and oxaloacetate. Like mammalian liver, the hepatopancreas of active snails appears to possess a β-carboxylase (see Table 8). It was not possible, however, to detect β-carboxylase activity in the hepatopancreas of the hibernating animal. The level of citrate synthesis was dependent on the ATP concentration, but added coenzyme A was without effect, and it appears therefore that the limiting factor in the cycle probably lies in the concentration of 'condensing enzyme'.

Judah & Rees (1953) have shown that in rat-liver preparations ATP will activate fatty acid oxidation. The inability of hepatopancreas preparations to oxidize added octanoate or acetate in the presence of Krebs's cycle intermediates and ATP suggests either that the tissue does not possess this oxidase or that, under the conditions of our experiments, this enzyme is destroyed.

The distribution of nitrogen in the hepatopancreas suspension showed that about 12 % of the nitrogen

of the tissue suspension was localized in the mitochondrial fraction. This may be contrasted with the 25% localized in the mitochondrial fraction of rat liver. The rate of oxidation of Krebs's cycle intermediates in the presence of hepatopancreas mitochondria was about 15% of the activities for rat-liver mitochondria. The Krebs's cycle activity of the mitochondria was of the order that would be expected from the activity of the whole suspension. The distribution of cytochrome oxidase and succinoxidase in the hepatopancreas suspension were similar to the results obtained by Hogeboom, Schneider & Pallade (1948) for rat-liver preparations.

The addition of co-factors, e.g. DPN, are without effect on the oxidation of cycle intermediates by rat-liver and rat-kidney mitochondria preparations (Judah, 1951). This is in marked contrast to the stimulatory effect of DPN on the oxidation of L-malate and α-ketoglutarate by hepatopancreas mitochondria.

In mammalian tissues the oxidation of Krebs's cycle intermediates is accompanied by the esterification of inorganic phosphate from the medium. The system used in our investigations was tested in rat-liver preparations and the P:O ratios found for succinate, L-glutamate and octanoate were of the same order as those reported in the literature. Esterification of inorganic phosphate took place at 28° in the whole 0·2M-sucrose hepatopancreas suspensions but the level of phosphate esterified was not increased by the co-oxidation of Krebs's cycle intermediates. Variation in the temperature of incubation, salt and sucrose concentrations and the addition of respiratory co-factors did not elicit oxidative phosphorylation. The observation that the phosphate esterification is not interrupted by the addition of dinitrophenol suggests that such esterification as is observed is not oxidative in nature. Under anaerobic conditions there was no decrease in the phosphate esterified. It appears likely that these phosphorylations are due to anaerobic glycolysis.

Krebs, Gurin & Eggleston (1952) have suggested that the Krebs's cycle may have a dual function. At higher levels of animal organization the cycle may function both as a mechanism for energy production and as a pathway for the synthesis of special molecules. At a lower level of organization the cycle may only possess the latter function. Such a hypothesis as proposed by Krebs may be an explanation of our failure to demonstrate phosphorylations associated with Krebs's cycle activity.

No variation in the oxidation of Krebs's cycle intermediates was observed in a comparison of preparations of the hibernating and active *H. pomatia*. All results were examined statistically and no significant differences were found. The absence of a β-carboxylase in the hibernating hepatopancreas could reduce cycle activity. With the loss of the ability to utilize galactose in the hibernating animal, there exist in the hepatopancreas during hibernation at least two mechanisms for reducing the utilization of carbohydrate.

SUMMARY

1. The oxidation of a number of Krebs's cycle intermediates by tissues suspensions and mitochondria of *H. pomatia* have been studied.

2. Reactions of the Krebs's cycle were demonstrated to occur.

3. The evidence for the presence of the cycle and its role in the hepatopancreas is discussed.

4. Differences in the metabolism of preparations from active as compared with hibernating animals are reported and discussed.

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Enzyme Formation in *Saccharomyces fragilis*

1. INVERTASE AND RAFFINASE

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The phenotype of an organism is a variable property which is dependent upon an interaction between its genotype and its environment and can be altered by changes in either of these. A necessary corollary of this is that the genotype can only be reliably deduced from the phenotype when either the environment remains constant or the influence of the environment on the phenotype is accurately known. Experimentally, it is no simple matter to maintain growing cells in a constant environment, and a detailed knowledge of the effect of the environment on the phenotype would appear to be an essential prerequisite for an understanding of the mechanism of the genic control of enzyme formation. It was with this aim in view that investigations into the factors affecting enzyme formation in *Saccharomyces fragilis* were begun. This paper will be chiefly concerned with the anaerobic utilization of sucrose and raffinose by this yeast.

Willstätter, Lowry & Schneider (1925) and De Ley & Vandamme (1951) have described increases in the invertase content of brewer's yeast following continuous addition of sucrose to the washed yeast suspended in a salt solution containing ammonium, potassium, magnesium, nitrate and phosphate ions. The former authors used as their starting material a yeast in which invertase activity had become attenuated during repeated fermentations in the brewery and they showed that the slow addition of sucrose gave a much bigger stimulation than a large single addition at the start. Glucose and maltose were almost as effective if added in the same way. The maximum increase in invertase activity which they obtained was about 14-fold. De Ley & Vandamme (1951) showed that invertase activity of the cells was greatest during the stationary phase of growth on a beer-wort medium and that the ability to form extra invertase on slow addition of sucrose was also greatest for cells harvested during the stationary phase. Omission of ammonium ions or phosphate resulted in a marked fall in the amount of invertase formed, whereas replacement of ammonium ions by asparagine resulted in increased invertase formation.

It is possible that the glucose-fructose linkage in raffinose is hydrolysed by invertase. However, the rate of hydrolysis is only about one-tenth that for sucrose by the same enzyme preparations. Adams, Richtmeyer & Hudson (1943), among others, considerably purified invertase from brewer's yeast and tested the preparations for ability to hydrolyse a number of di-, oligo-, and poly-saccharides. Their most highly purified preparations possessed β-fructofuranosidase, α-D-galactosidase, β-D-glucosidase, β-D-mannosidase and inulase activities. The ratio (rate of sucrose hydrolysis)/(rate of raffinose hydrolysis) varied in these experiments from 4·11 to 8·59, yet despite the fact that their preparations were obviously impure, the authors were of the opinion that sucrose and raffinose were hydrolysed by the same enzyme. There is, therefore, as has been stated by Neuberg & Mandl (1950), no certainty that hydrolysis of the glucose-fructose linkage in sucrose and raffinose is due to the activity of invertase. Recently, however, Gilliland (1949) and Winge & Roberts (1952) have shown that *Saccharomyces italicus* has three polymeric genes controlling the ability to ferment raffinose, and that the ability to ferment sucrose appears to be controlled by the same three genes. A considerable number of yeast hybrids was examined, and in every case fermentation of raffinose and rapid fermentation of sucrose

occurred together. Failure to ferment raffinose entailed failure to ferment sucrose rapidly. The raffinose non-fermenters could ferment sucrose very slowly, but this latter activity was correlated with the presence of a gene M_1 controlling the ability to ferment maltose by means of a non-specific α-glucosidase. Winge & Roberts (1952) appear to believe that a single enzyme, β-fructofuranosidase, is involved in both the rapid fermentation of sucrose and fermentation of raffinose. However, Hestrin & Lindegren (1950*a*, *b*) state that certain hybrid saccharomycetes yield extracts containing a sucrase which is inactive on raffinose and on maltose, and they are of the opinion that there may be a specific gluco-sucrase.

S. fragilis is a lactose fermenter and is completely unable to utilize maltose for growth, either aerobically or anaerobically. It would appear unlikely, therefore, that sucrose utilization by this yeast would be complicated by the possibility of hydrolysis by means of non-specific α-glucosidases, and this yeast should therefore be suitable material with which to investigate the relationship between sucrose and raffinose hydrolysis. The fermentation of raffinose by *S. fragilis* seems to be an adaptive property (Karström, 1930), whereas fermentation of sucrose is normally a constitutive property. A study of the conditions under which the ability to hydrolyse sucrose and raffinose can be acquired might, therefore, be expected to yield information concerning the identity or otherwise of the enzymes involved.

METHODS

Organism, growth media and cell suspensions. The organism used throughout this work was a strain of *S. fragilis* Jörgensen obtained from the Centraalbureau voor Schimmelcultures at Delft. The basal medium was that of Davies, Falkiner, Wilkinson & Peel (1951) and contained ammonium and potassium phosphates, trace metals and Difco yeast extract. To this was added an appropriate source of carbon as indicated in the following paragraphs. The basal medium and glucose, fructose and galactose solutions were sterilized in the autoclave; sucrose, lactose, raffinose and melibiose were sterilized by filtration. In one experiment (Fig. 1) a synthetic medium was used having a composition similar to that of the basal medium but with the Difco yeast extract replaced by the following amounts of growth factors/ml. medium: biotin, 10^{-4} μg.; calcium pantothenate, 0·03 μg.; pyridoxine, nicotinic acid, inositol and thiamine, 0·33 μg. With the exception of thiamine, which was sterilized by filtration, the growth factors were added to the rest of the medium before sterilization. For growth, the medium was dispensed into Roux bottles (150 ml./bottle), inoculated with 0·5 ml. of a 24 hr. culture grown on basal medium plus 2% (w/v) glucose (unless stated otherwise), and the bottles incubated on their sides at 25°. For the 'age of culture' experiments two sets of seven Roux bottles each were inoculated from the same culture with an interval of 12 hr. between each set. The stock culture was held at 4° in the interval between the two sowings. After incubating for the required time a suitable number of bottles was removed and the yeast harvested.

For enzymic tests the yeast was harvested on the centrifuge, washed twice with distilled water, and suspended in distilled water to a suitable density. The optical densities of the suspensions were measured with a Hilger absorptiometer and converted to mg. dry matter/ml. by means of a calibration curve. All experiments were carried out at 25°. Where concentrations of solutes are given as % this means % (w/v) in all cases.

Estimation of invertase and raffinase activity. This was usually determined by measuring the increase in reducing sugar when cell suspensions or extracts were incubated in test tubes at 25° with sucrose or raffinose in the following system: 1·5 ml. K_2HPO_4-citric acid buffer, pH 4·5 (prepared according to the formula of McIlvaine (1921) but using K_2HPO_4 in place of Na_2HPO_4), 1·0 ml. 10% sucrose, 0·25 ml. 1% cetyltrimethylammonium bromide (CTAB), cell suspension or preparation and distilled water to a total volume of 4·0 ml. Duplicate tubes were set up containing all the components except the sugar, and at zero time the sugar was added to one tube. At the end of the incubation period, usually 30 min., 1·0 ml. of 5% $ZnSO_4$, $7H_2O$ and 1·0 ml. of 0·3 N-$Ba(OH)_2$ were added to both tubes and the sugar to the control. The precipitate was removed immediately by centrifugation and a sample of the supernatant analysed for reducing sugar. Conditions were so chosen that the amount of reducing sugar formed did not exceed 2 μmoles/30 min.; under these conditions the rate of formation of reducing sugar is proportional to the amount of enzyme present. Enzyme activities are expressed as μmoles sugar hydrolysed/mg. dry wt. yeast/hr., and one enzyme unit is defined as that amount of enzyme which will hydrolyse 1·0 μmole of sucrose or raffinose in 1 hr. at 25° under the above conditions.

When determining the enzyme content of whole cells there is always the possibility that permeability effects may be a controlling factor and the total enzyme content of the cell may not be estimated. CTAB at a suitable concentration removes these permeability barriers (Salton, 1951). At the CTAB concentration used in the tests described above fermentation of sucrose and raffinose is abolished but hydrolysis is unaffected. Although in this particular case the presence of CTAB has little effect on the measured invertase activity, other enzymic activities (e.g. lactase activity in *S. fragilis*, Davies, unpublished) are greatly increased in the presence of CTAB. Consequently, CTAB has been added as a routine to the invertase test system. In some of the earlier work iodoacetate at a final concentration of 0·01 M was used to inhibit fermentation, but it was subsequently found to cause some inhibition (10%) of hydrolysis. Two experiments are described in which iodoacetate was used for invertase estimations (Table 2 and Fig. 4), but the fact that it is somewhat inhibitory does not affect the conclusions drawn from the experiments.

Estimation of reducing sugar. The methods of Shaffer & Somogyi (1933) and of Nelson (1944) were used. In the case of Nelson's method the heating time was increased to 45 min., since 20 min. was found to be insufficient for maximum reduction. Iodoacetate and CTAB were found to be without effect on the estimations at the concentrations used.

Cell preparations. Preparations of broken cells were made by (*a*) shaking with glass beads (Ballotini no. 12) in the Mickle disintegrator (Mickle, 1948), (*b*) grinding with alumina according to McIlwain (1948). Extracts were pre-

pared by centrifuging the broken cell preparations at 1000 *g* for 15 min. Acetone powders were prepared by the method of Epps (1944).

Adaptation in washed-cell suspensions. Invertase and raffinase formation in washed-cell suspensions was studied by incubating 2 vol. of yeast suspension (about 10 mg. dry wt. cells/ml.), 3 vol. of 0·2 M-K_2HPO_4-citric acid buffer (usually pH 4·5) and 2 vol. of 10% sugar in 6 × 1 in. test tubes or 100 ml. centrifuge tubes, depending on the volume of the mixture. The gas phase was air. Replacement of the air by N_2 had no effect on either the rate or the extent of the adaptation. At the end of the adaptation period the cells were recovered by centrifugation, washed twice with distilled water and suspended in distilled water to a suitable density.

Fermentation rates. These were measured by the usual Warburg manometric techniques in N_2 at 25° as described by Umbreit, Burris & Stauffer (1949), and are expressed throughout as $Q_{CO_2}^{N_2}$ = μl. CO_2/mg. dry wt. cells/hr.

Sugars. With the exception of melibiose (Kebo, Stockholm), Kerfoot's bacteriological sugars were used throughout without purification.

RESULTS

Adaptive fermentation of sugars by Saccharomyces fragilis

Washed-cell suspensions prepared from 20 hr. cultures of *S. fragilis* in basal medium plus 2% glucose ferment only glucose and sucrose when incubated in phosphate buffer at pH 5·0. Galactose, lactose and raffinose are not fermented even after 6 hr. incubation. If either galactose or lactose is substituted for glucose in the growth medium then both galactose and lactose, in addition to glucose and sucrose, are fermented by the suspensions, but raffinose is fermented only very slowly. If raffinose is substituted for glucose in the growth medium then suspensions of cells prepared therefrom rapidly ferment raffinose but not galactose or lactose (Table 1). The ability to ferment galactose, lactose or raffinose would seem to be an adaptive property (Karström, 1930), whereas ability to ferment glucose or sucrose is a constitutive property in *S. fragilis*. However, Fig. 1 shows that the fermentation of sucrose by cells grown in basal medium plus 2% glucose does not parallel that of glucose with respect to the attainment of maximum rate. For glucose this is achieved within 10 min., but for sucrose the time required is about 2 hr. Similar results were obtained with cells grown in synthetic medium containing 10% glucose. If sucrose replaces glucose in the growth medium then the maximum rate of sucrose fermentation is achieved within 10 min. also (Fig. 1). This suggests that the

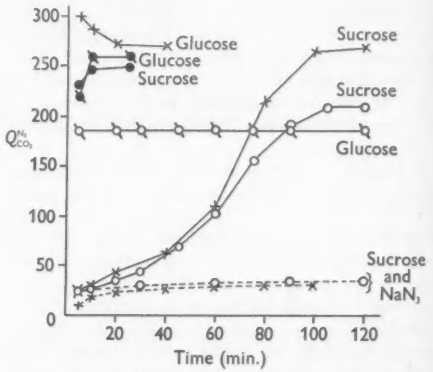

Fig. 1. Fermentation of glucose and sucrose by washed suspensions of *S. fragilis*. Warburg manometers contained in a total vol. of 3·5 ml.: 0·057 M-phosphate buffer, pH 5·0, 0·084 M-sugar, 2·5 mg. dry wt. of cells and, where added, 3×10^{-4} M-NaN_3. Gas phase: N_2. ○—○ and ○—○, grown in basal medium + 2% glucose; ●—● and ●—●, grown in basal medium + 2% sucrose; ×—×, grown in synthetic medium + 10% glucose.

ability to ferment sucrose is partially adaptive in character. The adaptive enzymes involved in galactose and lactose fermentation are not readily synthesized anaerobically in suspensions prepared from cells grown in basal medium plus 2% glucose, since they do not appear within 6 hr., even when amino acids and small amounts of glucose are added (cf. Spiegelman, 1946; Monod, 1947; Pollock & Wainwright, 1948).

S. fragilis will grow to a limited extent on basal medium alone, and washed suspensions prepared from cells grown in this way exhibit quite a different

Table 1. *Adaptive fermentation of galactose, lactose and raffinose by* S. fragilis

(*S. fragilis* grown on basal medium plus 2% sugar for 24 hr. at 25°. Warburg manometers contained, in a volume of 3·5 ml.: 0·057 M-phosphate buffer pH 5·0, 0·084 M-sugar, 2·5 mg. dry wt. of yeast. Gas phase: N_2. Activities as μl. CO_2/mg. dry wt. of cells/hr.)

Additions to basal medium	Substrate for fermentation				
	Glucose	Sucrose	Galactose	Lactose	Raffinose
Glucose	233	240	(3)*	(3)	(3)
Sucrose	256	245	(2)	(1)	20
Galactose	154	197	233	240	10
Lactose	182	185	173	230	11
Raffinose	177	163	0	0	164

* The figures in brackets are probably not significantly different from zero.

pattern of sugar fermentation from that described above. Glucose and sucrose are fermented at similar rates immediately, but the rates increase to 3 or more times their initial values over a period of 2–3 hr. Galactose, lactose and raffinose are fermented very slowly at first and the rates increase for 5–6 hr. until, for lactose and raffinose, they approximate to that for glucose. The increases in rate do not occur in the presence of 3×10^{-4} M-sodium azide, except in the case of glucose and sucrose, but even here the final rate is considerably less than that reached in the absence of azide (Fig. 2). Occasionally the increase in rate of glucose fermentation is also prevented by azide. The increase in rate of sucrose fermentation observed with cells grown in basal medium plus 2 % glucose is

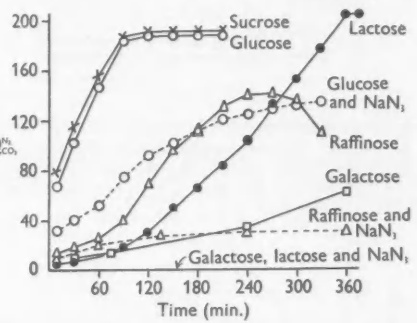

Fig. 2. Fermentation of sugars by washed suspensions of *S. fragilis* grown in basal medium alone. Conditions as for Fig. 1.

also prevented by 3×10^{-4} M-azide (Fig. 1). This effect of azide is similar to that described by Spiegelman (1947) for galactose and maltose fermentation by *S. cerevisiae*. Lactose fermentation by *S. fragilis* is inhibited to the extent of 30 % by 3×10^{-4} M-azide and it cannot be definitely concluded that adaptation is inhibited by azide in this case.

The increase in the rate of fermentation of glucose shown by cells which have been grown on basal medium alone suggests that part of the increase in the rate of galactose, lactose and raffinose fermentation might be due to a general rise in the activity of that part of the glycolysis system that is common to all sugars. This possibility is supported by the results of the following experiment. Suspensions of *S. fragilis*, grown in basal medium and showing a low initial rate of fermentation of glucose ($Q^{N_2}_{CO_2} = 43$ in 0·1 M-phosphate, pH 5·0), were incubated in 0·15 M-glucose–0·1 M-phosphate, pH 5·0, until the fermentation rate was at a maximum ($Q^{N_2}_{CO_2} = 189$). The cells were recovered, washed in water and the rates of fermentation of galactose and lactose measured under the same conditions. The $Q^{N_2}_{CO_2}$ for galactose had risen from 9 to 27 and for lactose from 3 to 35.

It is clear from these experiments that the composition of the growth medium affects not only the enzymic composition of the cells, but also the subsequent ability of the cells to acquire new activities. Following growth for 24 hr. in basal medium plus 2 % glucose, the cells are unable to ferment galactose, lactose and raffinose and are unable to adapt to ferment these sugars within 6 hr. The ability to ferment sucrose is initially low, but adaptation can occur rapidly. If growth takes place in basal medium alone, then the resulting cells ferment galactose, lactose and raffinose at a very low initial rate but can now adapt with varying degrees of rapidity, while fermentation of sucrose proceeds at a rate equal to that of glucose.

Fermentation and hydrolysis of sucrose

The plot of fermentation rate against time for sucrose fermentation by *S. fragilis* grown in basal medium plus 2 % glucose gives the typical S-shaped curve described by other workers for the formation of adaptive enzymes (e.g. Spiegelman, 1946), and the effect of azide is consistent with the view that adaptive enzyme formation is involved. Table 2 (*a*) shows that the increase in rate of sucrose fermentation is in fact accompanied by an increase in invertase activity, and that this increase does not occur in presence of azide (Table 2*b*). During the adaptation there is an increase in cell substance, as determined turbidimetrically, and also an increase in cell numbers which is of borderline significance (Table 3). Similar increases also occur when glucose replaces sucrose, though in this case very little invertase is formed and therefore the changes in cell substance may be unrelated to invertase formation. If it is assumed that the new invertase is formed only in the new cell substance, then this would have an invertase content of 44 units/mg. dry wt., a value which is by no means impossible since values as high as 150 have been observed for this yeast (see Table 9).

Attempts were made by various means to obtain preparations from unadapted cells having activities approaching those of adapted cells, but in no case was this successful (Table 4).

Rate of formation of invertase by cell suspensions. This is shown in Fig. 3. The curve is of the typical S-shape characteristic of many adaptive-enzyme systems. The 'growth rate' constant for invertase formation (see Table 7), calculated from the linear portion of the ln (invertase units) plot (Fig. 3), is 2·03, which is very similar to the value of 2·87 for maltase formation calculated from the figures given by Halvorson & Spiegelman (1952).

Effect of pH *on invertase formation by cell suspensions.* A washed-cell suspension of density 8·0 mg. dry wt./ml. was prepared from a 22 hr. culture in basal medium plus 10% glucose, and samples were incubated with sucrose in citrate-phosphate buffers of the appropriate pH for 1·5 hr. The cells were recovered, washed twice with distilled water and suspended in water at a density of

Table 2. *Formation of invertase during adaptation to sucrose by washed-cell suspensions of* S. fragilis

(Fermentation rates measured as in Table 1. Adaptations carried out by incubating 2 vol. of yeast suspension (10 mg. dry wt. cells/ml.) with 3 vol. of 0·2 M-K_2HPO_4-citric acid buffer pH 4·5 and 2 vol. of 10% (w/v) sucrose in air at 25°, without shaking, for the times indicated below. The cells were then recovered, washed twice with distilled water and resuspended in distilled water. Invertase content of the cells was estimated by measuring the increase in reducing sugars when the cell suspension was incubated with sucrose at pH 4·5 using iodoacetate as inhibitor of fermentation. Invertase units/mg. dry wt. = μmole sucrose hydrolysed/mg. dry wt. yeast/hr.)

(*a*) *Correlation between invertase content and rate of sucrose fermentation*

(Organism grown in basal medium + 2% glucose for 19 hr.)

Period of adaptation (min.)	Q_{CO_2} (glucose)	Q_{CO_2} (sucrose)	Invertase content (units/mg. dry wt.)
0	225	36	1·4
30	249	194	4·1
90	252	302	8·4

(*b*) *Inhibition of invertase formation by* 10^{-3} M-NaN_3

(Organism grown in basal medium + 10% glucose for 20 hr. Period of adaptation 1·5 hr.)

Sugar present in adaptation system	Invertase content (units/mg. dry wt.) Intact cells	Smashed cells	Extract
None	0·12	0·12	0·18
Glucose	0·47	—	—
Sucrose	3·51	3·40	3·51
Sucrose + 10^{-3} M-NaN_3	0·23	0·18	0·18
Initial invertase	0·18	0·18	0·18

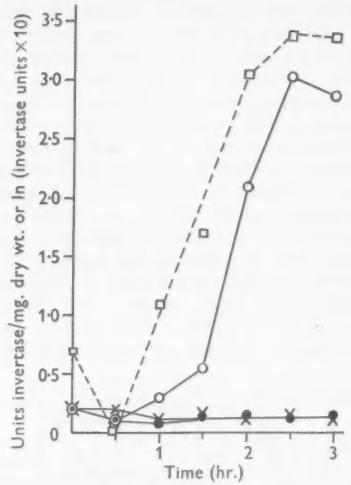

Fig. 3. Invertase formation by washed-cell suspensions of *S. fragilis*. Cell suspensions incubated with citrate-phosphate buffer, pH 4·5, ●—●; buffer plus sucrose, ○—○; buffer plus glucose ×—× as described in Table 2. □—□, ln (invertase units).

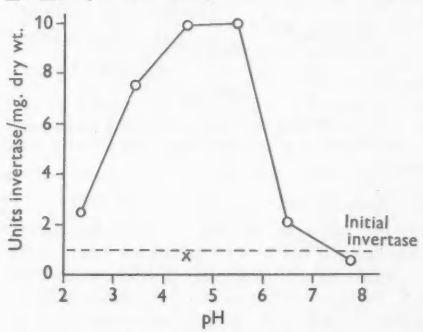

Fig. 4. Effect of pH on invertase formation by suspensions of *S. fragilis*. Cell suspensions incubated for 1·5 hr. with sucrose in citrate-phosphate buffer of the appropriate pH as described in Table 2. Invertase estimated in presence of 0·01 M-iodoacetate. × = Invertase content when incubated with glucose at pH 4·5.

Table 3. *Changes in dry matter and in total cell numbers during adaptation to sucrose in washed cell suspensions*

(Adaptations and invertase estimations carried out as in Table 2; period of adaptation 2·5 hr.; invertase content expressed as units/mg. dry wt. cells. Dry matter estimated turbidimetrically. Each cell count is the mean of nine separate determinations.)

Sugar present in adaptation system	Invertase content Initial	Final	Dry matter (mg./ml.) Initial	Final	Cell numbers × 10^{-5} Initial	Final
None	0·50	0·53	1·61	1·67	145 ± 20	146 ± 20
Glucose	0·50	0·70	1·57	2·06	143 ± 20	159 ± 20
Sucrose	0·50	7·0	1·60	1·88	137 ± 20	164 ± 20

Table 4. *Effect of various treatments on invertase activities of unadapted and adapted* S. fragilis

(The yeast was grown in basal medium + (*a*) 10% glucose for 24 hr., (*b*) 2% glucose for 42 hr., (*c*) 2% glucose for 16 hr. for unadapted yeast or 1% raffinose for adapted yeast. In (*a*) the adapted cells were obtained by incubating the unadapted cells with sucrose and buffer for 2 hr. as described in Table 2. For the invertase estimations CTAB was omitted from the normal test system. Treatments were carried out as follows: (1) *CTAB*—0·25 mg. CTAB/mg. dry wt. yeast was added to the yeast suspension and the invertase activity measured; (2) *Mickle*—10 ml. suspension (116 mg. dry wt. yeast) was shaken with glass beads in the Mickle disintegrator for 30 min. and invertase estimated in the resulting preparation; (3) *acetone*—10 ml. suspension (116 mg. dry wt. yeast) was added rapidly to 80 ml. acetone at 0° and allowed to stand at 0° for 20 min., cells were recovered, washed with acetone and ether and dried *in vacuo*; (4) *alumina*—yeast equivalent to 232 mg. dry wt. ground with alumina as described by McIlwain (1948) and 0·2 M-K_2HPO_4-citric acid buffer, pH 4·5, added to give a total vol. of 20 ml.; invertase estimated on the resulting mixture.)

	Treatment given to suspension				
Kind of cells	None	CTAB	Mickle	Acetone	Alumina
(*a*) Unadapted	0·40	0·50	0·56	0·38	—
Adapted	4·55	4·80	4·69	5·10	—
(*b*) Adapted	18·7	18·3	18·2	14·7	15·7
(*c*) Adapted	—	108·2	—	—	—
Unadapted	0·09	0·28	0·28	—	—
Unadapted plus boiled extract of adapted cells	—	0·22	0·28	—	—

Table 5. *Effect of acetate on sucrose fermentation and on adaptation to sucrose by cell suspensions*

(Fermentation rates were measured on the adapted cells as in Table 1. The yeast was grown in basal medium + 10% glucose for 18 hr. For (*a*) adaptation was carried out in sucrose + 0·2 M-phosphate buffer, pH 5·0; for (*b*) adaptation was carried out in N_2 in Warburg manometers containing 0·084 M-sucrose, 0·2 M-buffer (phosphate or acetate) of the appropriate pH, and 2·1 mg. dry wt. of yeast in a total volume of 3·5 ml. The values for $Q_{CO_2}^{N_2}$ at pH 6·0 have been corrected for retention of CO_2.)

		(*a*) $Q_{CO_2}^{N_2}$		(*b*) $Q_{CO_2}^{N_2}$ (sucrose)	
pH	Buffer	Glucose	Sucrose	Initial	After 2·5 hr. adaptation
6·0	Phosphate	286	217	7	232
	Acetate	300	260	6	101
5·5	Phosphate	300	242	7	228
	Acetate	294	240	6	39
5·0	Phosphate	295	255	12	234
	Acetate	100	88	4	20
4·5	Phosphate	268	250	—	—
	Acetate	40	48	4	4

Table 6. *Inhibition of adaptation to sucrose by acetate*

(The yeast was grown in basal medium + 10% glucose for 17 hr. Adaptation was carried out in N_2 in Warburg manometers containing 2·5 mg. dry wt. of cells, 0·084 M-sucrose, 0·086 M-phosphate buffer pH 5·5, potassium acetate buffer, pH 5·5, to give the acetate concentrations indicated in the table, and sufficient 0·2 M-KCl to give 0·143 M-K^+ in all manometers.)

	Acetate concentration (M)					
	0	0·011	0·023	0·034	0·046	0·057
$Q_{CO_2}^{N_2}$ (glucose), initial	153	154	157	164	167	161
$Q_{CO_2}^{N_2}$ (sucrose), initial	8	8	8	8	5	5
$Q_{CO_2}^{N_2}$ (sucrose), after 2 hr.	132	140	69	42	27	19

4·0 mg. dry wt./ml. Invertase was determined using iodoacetate as inhibitor of fermentation. The result is given in Fig. 4. Maximum invertase is formed at pH 4·5–5·5. The same result was obtained if the rate of increase of $Q_{CO_2}^{N_2}$ (sucrose) was used as the measure of adaptation.

Effect of acetate on sucrose fermentation. In the course of the experiments just described, sodium acetate buffers were used to extend the pH range beyond that of the KH_2PO_4-Na_2HPO_4 system. It was found that for values of pH below 5·5 the rate of fermentation of glucose or sucrose was markedly

different in the two buffer systems. The rate rapidly diminished in acetate buffer below pH 5·5. Development of the ability to ferment sucrose is also impaired in acetate buffers when compared with the corresponding phosphate buffers (Table 5). Citrate-phosphate buffers give the same results as phosphate buffers. Failure to adapt in acetate buffer at pH 5·5 is due neither to lack of phosphate nor of potassium ions, since the inhibition still occurs in mixed acetate-phosphate buffer at this pH. At pH 5·5 the fermentation rates for glucose and sucrose are the same in either buffer. If the concentrations of phosphate and potassium ions and the ionic strength are held constant then, at pH 5·5, the ability to adapt is inversely related to the acetate concentration (Table 6). Adaptation does occur in acetate buffers at, or above, pH 6·0, and it would seem possible that the lack of adaptation is due to inhibition by undissociated acetic acid.

Fig. 5. Effect of 'age of culture' on ability of washed suspensions of *S. fragilis* to form invertase. The yeast was grown in basal medium + 10% glucose and adaptation was carried out at pH 4·5 for 2 hr. as described in Table 2. Curve *A* = invertase content of cells/mg. dry wt. before adaptation; curve *B* = same after adaptation; curve *C* = growth of cells in mg. dry wt./ml. culture.

Effect of time of growth on ability to adapt. The extent to which adaptive formation of invertase occurs in cell suspensions incubated with sucrose and buffer varies with the time for which the cells have been grown (Fig. 5). Adaptation takes place most readily in young cells growing at their maximum rate and falls off as the growth rate decreases until, towards the end of growth, the cells cease to respond to sucrose. These observations are in agreement with those of Hegarty (1939) on adaptation to galactose by *Streptococcus lactis* and the opposite of those of De Ley & Vandamme (1951) for invertase formation by a bottom yeast.

Effect of time of growth on invertase content of cells grown in basal medium plus various sugars. (*a*) 2% Glucose. Occasional batches of cells which had been grown for longer than 24 hr. on this medium were found to ferment sucrose at the maximum rate without an initial period of adaptation. An investigation of the variation of invertase content of the cells with time of growth gave the results shown in Fig. 6. The invertase content remains low throughout the early, rapid phase of growth, and only starts to increase when growth slows down after about 18 hr. It increases very rapidly about the time that growth ceases. The values for the total intracellular invertase present in each Roux bottle are also shown in Fig. 6, and it will be seen that this increases continuously throughout the growth period.

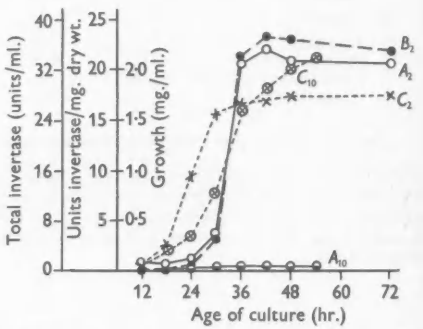

Fig. 6. Effect of 'age of culture' on invertase content of *S. fragilis* and on total intracellular invertase formed during growth. The yeast was grown in basal medium + (*a*) 2% glucose, or (*b*) 10% glucose, the inoculum being taken from a 24 hr. culture in basal medium + 2% glucose. Total intracellular invertase content of culture/ml. = (invertase/mg. dry wt. cells) × (dry wt. cells/ml. culture). Curves A_2 and A_{10} = invertase content of cells/mg. dry wt.; curve B_2 = total intracellular invertase/ml. culture; curves C_2 and C_{10} = growth of cells/ml. culture. Subscripts indicate the glucose content of the growth medium.

(*b*) 10% Glucose. With 10% glucose in the growth medium the invertase content of the cells remains low throughout the growth period of 54 hr. (Fig. 6, curves A_{10} and C_{10}).

(*c*) 2% Sucrose. The results with 2% sucrose in the growth medium are shown in Fig. 7. It was expected that the invertase content of the cells would reach a high level in the early stages of growth and would remain high, possibly falling after growth had ceased. The observed behaviour is quite different. The enzyme content is certainly high in very young cells, but during the period of maximum growth rate it rapidly falls, to rise again towards the end of growth. The total amount of intracellular invertase present in the culture is seen to increase throughout the growth period. The

variation in invertase content per unit mass of cells is therefore due to a difference between the rate of invertase synthesis and the rate of cell growth. This is more clearly seen in Table 7, where the 'growth rates' for cell substance and invertase are compared for cultures grown on basal medium plus 2% glucose or 2% sucrose. In the very early stages of growth on sucrose, following inoculation with 24 hr. cells grown on 2% glucose, the cells of the inoculum probably become adapted in the same way as do washed-cell suspensions incubated with buffered sucrose solutions. Hence the high invertase content of the very young cells. During the logarithmic growth phase, and for some time after, cell growth proceeds faster than invertase synthesis, the latter in fact decreasing. In the later stages the cell growth rate falls but the 'growth rate' for invertase increases again up to the point where growth ceases, when it drops to zero. When the growth medium contains 2% glucose the 'growth rate' for invertase does not show the fall in the middle periods which is found in the sucrose medium under the conditions given in Table 7*b*.

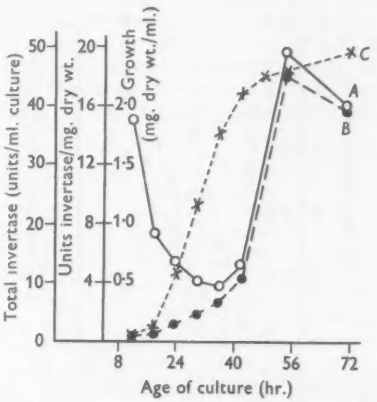

Fig. 7. Effect of 'age of culture' on invertase content of *S. fragilis* and on total intracellular invertase formed during growth on basal medium + 2% sucrose. The inoculum was as for Fig. 6. Curve *A* = invertase content of cells/mg. dry wt.; curve *B* = total intracellular invertase/ml. culture; curve *C* = growth of cells in mg. dry wt./ml. culture.

(*d*) 1% Raffinose. The failure of cells growing in basal medium plus 10% glucose to synthesize invertase might be interpreted as an inhibition of synthesis by glucose. The other growth experiments give no clear evidence in support of this. However, evidence which strongly suggests that glucose may be inhibitory of invertase formation was obtained from an examination of the invertase content of cells growing in basal medium plus 1% raffinose. The results are shown in Fig. 8. In this case the invertase content of the cells increases rapidly to reach a peak value at the end of the logarithmic growth phase which is almost 20 times higher than the maximum value found for cells growing in 2% sucrose medium. In the later stages of growth the invertase content falls somewhat, but is still about 10 times higher than the value for cells grown in 2% sucrose medium. The total intracellular invertase content of the culture increases more or less in parallel with cell growth. This great increase in invertase is not to be ascribed specifically to an

Table 7. *'Growth rates' for cell substance and invertase for* S. fragilis *growing on (a) glucose, (b) and (c) sucrose*

(The yeast was grown in basal medium plus (*a*) 2% glucose, (*b*) and (*c*) 2% sucrose. In (*a*) and (*b*) the media were inoculated from a 24 hr. culture in basal medium plus 2% glucose; in (*c*) the inoculum was from a 24 hr. culture in basal medium plus 10% glucose. Invertase was determined in presence of CTAB.)

$$\text{Growth rate (cell substance)} = \frac{1}{t_2 - t_1} \ln \frac{\text{(mg. dry wt. cells/ml. culture) at } t_2}{\text{(mg. dry wt. cells/ml. culture) at } t_1},$$

$$\text{'Growth rate' (invertase)} = \frac{1}{t_2 - t_1} \ln \frac{\text{(total intracellular invertase/ml. culture) at } t_2}{\text{(total intracellular invertase/ml. culture) at } t_1}.$$

Period of growth (hr.)	'Growth rate' (*a*) Cell substance	(*a*) Invertase	(*b*) Cell substance	(*b*) Invertase	(*c*) Cell substance	(*c*) Invertase
0–12	0·24	0·19	0·20	0·39	0·19	0·24
12–18	0·22	0·185	0·25	0·12	0·21	0·12
18–24	0·24	0·34	0·20	0·15	0·26	0·29
24–30	0·085	0·26	0·12	0·08	0·13	0·33
30–36	0·01	0·31	0·065	0·05	0·06	0·39
36–42	0·0	0·015	0·03	0·09	0·004	0·08
42–48	0·007	0·0	0·01	0·14	0·006	0·02
48–54	0·004	0·0	0·004	0·10	0·0	0·003

effect of raffinose, since growth in the basal medium alone also yields cells with a high invertase content. Thus, *S. fragilis* harvested after 18 hr. growth in basal medium alone has an invertase content of 132 units/mg. dry wt., compared with 7·3 for cells grown in basal medium plus 2 % sucrose and 138 for cells grown in basal medium plus 1 % raffinose.

Effect of the inoculum on the course of invertase formation. It is conceivable that the invertase content of cells growing in a sucrose medium may be affected by the initial invertase content of the inoculum. In the experiments of Fig. 7 and Table 7 the inoculum was taken from a 24 hr. culture on 2 % glucose-basal medium and the cells had an invertase

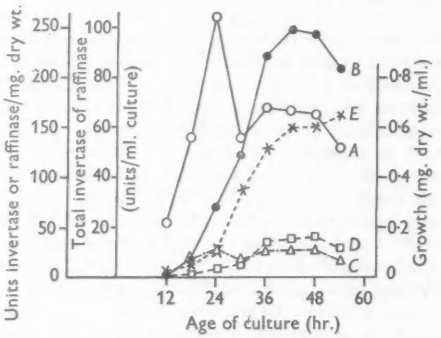

Fig. 8. Effect of age of culture on invertase and raffinase content of *S. fragilis* and on total intracellular invertase and raffinase formed during growth on basal medium + 1 % raffinose. The inoculum was as for Fig. 6. Curve A = invertase content of cells/mg. dry wt.; curve B = total intracellular invertase/ml. culture; curve C = raffinase content of cells/mg. dry wt.; curve D = total intracellular raffinase/ml. culture; curve E = growth of cells in mg. dry wt./ml. culture.

content of 1·4 units/mg. dry wt. The experiment was repeated using an inoculum taken from a 24 hr. culture in basal medium plus 10 % glucose, in which the cells had an invertase content of 0·28 unit/mg. dry wt. Such cells are able to form invertase when incubated in phosphate buffer plus sucrose (see Fig. 5). The results obtained are given in Fig. 9 and Table 7*c*. The initial rise in invertase content is now absent and the 'growth rate' for invertase increases throughout the growth period. In fact, the behaviour is similar to that found when growth takes place on glucose instead of sucrose. When the inoculum is grown in basal medium plus 2 % sucrose (invertase content of inoculum 7·0 units/mg. dry wt. cells) the variation of invertase content with time of incubation follows a pattern similar to that obtained when the inoculum is taken from a 2 % glucose culture (Fig. 10). It is clear from these experiments that the changes in invertase content during growth on 2 % sucrose medium are markedly influenced by the history of the inoculum used. It is by no means impossible that similar effects occur with other enzymes.

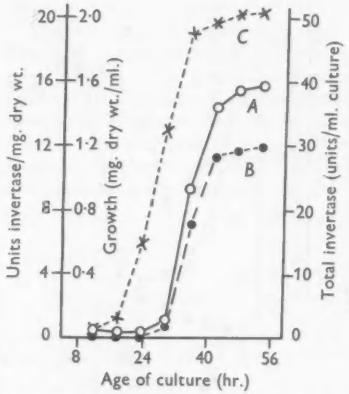

Fig. 9. As for Fig. 7, but inoculum taken from a 24 hr. culture in basal medium + 10 % glucose. Curve A = invertase content of cells/mg. dry wt.; curve B = total intracellular invertase/ml. culture; curve C = growth of cells in mg. dry wt./ml. culture.

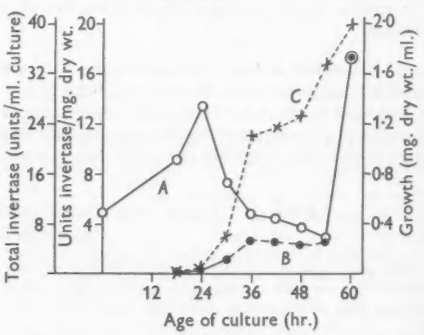

Fig. 10. As Fig. 7, but inoculum taken from an adapted culture grown for 15 hr. in basal medium + 2 % sucrose. Curve A = invertase content of cells/mg. dry wt.; curve B = total intracellular invertase/ml. culture; curve C = growth of cells in mg. dry wt./ml. culture.

Stimulation of invertase formation in washed suspensions of S. fragilis *by sugars other than sucrose.* The ability of a number of sugars to substitute for sucrose in stimulating the formation of invertase in washed cell suspensions was investigated using the test system described in the Methods section. With a sugar concentration of 2·86 % there was no significant stimulation by glucose, raffinose, lactose, galactose or melibiose, although fructose appeared to support some synthesis. The failure of raffinose to

stimulate is noteworthy, since raffinose is generally believed to be hydrolysed by invertase, and is in fact hydrolysed by cell suspensions of *S. fragilis* at about one-tenth the rate of sucrose. An investigation of the influence of sugar concentration on invertase formation under these conditions shows that the stimulation is not specific for sucrose. Fig. 11 shows the relationship between sugar concentration and extent of adaptation for sucrose, glucose, fructose and raffinose. Low concentrations of glucose and

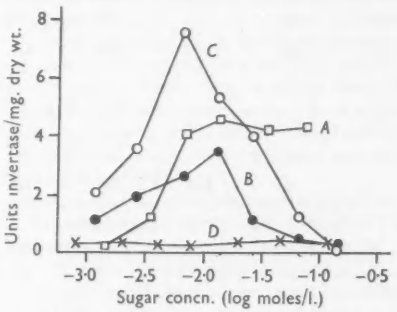

Fig. 11. Effect of sugar concentration on stimulation of invertase formation by washed-cell suspensions of *S. fragilis*. The yeast was grown in basal medium + 10% glucose for 18 hr. Adaptation was carried out at pH 4·5 for 3 hr. as described in Table 2. Curve *A* = sucrose; curve *B* = glucose; curve *C* = fructose; curve *D* = raffinose.

fructose are as effective as sucrose in stimulating invertase formation, but high concentrations are strongly inhibitory. Willstätter *et al.* (1925) showed that the slow addition of glucose was as effective as that of sucrose in stimulating invertase formation in a brewing yeast.

Fermentation and hydrolysis of raffinose

Quantitative estimation of the carbon dioxide liberated when raffinose was fermented to completion by washed-cell suspensions of *S. fragilis* in phosphate buffer at pH 4·5 and in the presence of 3×10^{-4} M-azide, gave the value 1·83 moles carbon dioxide/mole raffinose fermented. This suggests that the products of raffinose fermentation by this yeast are ethanol, carbon dioxide, fructose and melibiose. Melibiose cannot be metabolized by *S. fragilis*, and it has been shown by paper chromatography (Diding, unpublished results) that melibiose accumulates in the medium during growth of the yeast on basal medium plus 1% raffinose, and also during the fermentation of raffinose by washed suspensions of the organism. No sugars other than raffinose, fructose and melibiose could be detected.

Fermentation of raffinose by washed-cell suspensions. It has been shown (Fig. 2) that washed-cell suspensions of *S. fragilis* grown in basal medium alone slowly acquire the ability to ferment raffinose and that this response does not occur in the presence of 3×10^{-4} M-azide. On the other hand, fermentation of raffinose by cells grown in its presence is unaffected by this concentration of azide (Fig. 12). These observations would suggest that the increase in fermentation rate is due to an adaptive increase in some part of the raffinose fermenting system, although the situation is obviously complicated by the fact that the rate of fermentation of glucose also increases. However, in an experiment designed to examine the effect of adaptation to raffinose in cell suspensions on the subsequent ability to ferment

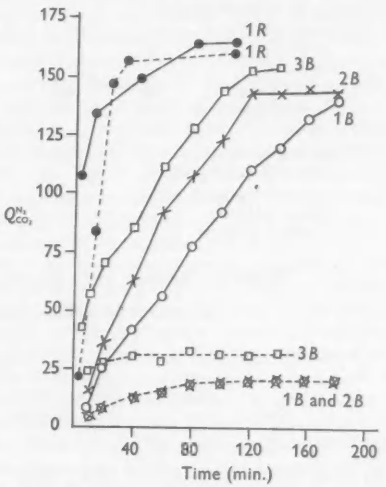

Fig. 12. Fermentation of raffinose, glucose and fructose by washed-cell suspensions of *S. fragilis* grown in basal medium alone or + 2% raffinose. Warburg-manometer contents as in Fig. 1. $NaN_3 = 10^{-3}$ M. Full lines = no NaN_3; broken lines = + NaN_3. *B* = cells grown in basal medium alone; *R* = cells grown in basal medium + 2% raffinose. Curve 1 = raffinose fermentation; curve 2 = glucose fermentation; curve 3 = fructose fermentation.

glucose, galactose and lactose, it was found that the rate of fermentation of raffinose by the adapted cells fell to about one-fourth to one-fifth when they were washed twice in distilled water. Glucose fermentation was not affected. Washing in 0·067 M-potassium dihydrogen phosphate, with or without 0·01 M-cysteine, had no protective effect. Removal of the cells by centrifugation and resuspension in buffer without washing also produced the drop in activity (Table 8). The washed cells gradually regained their activity when incubated with raffinose for 2–3 hr. That removal of an exogenous raffinase is not the explanation of these effects was shown by incu-

Table 8. *Effect of washing on fermentation of raffinose and glucose*

(The yeast was grown in basal medium alone for 48 hr. For fermentation rates Warburg manometers contained in a total vol. of 3·5 ml.: 0·086 M-phosphate buffer, pH 5·0, 0·084 M-sugar, 2·5 mg. dry wt. cells; gas phase, N_2. Adaptation to raffinose carried out as described for sucrose in Table 2.)

Treatment of suspension	$Q_{CO_2}^{N_2}$ Expt. I Raffinose	Expt. II Raffinose	Expt. II Glucose	Expt. III Raffinose	Expt. IV Raffinose	Expt. IV Supernatant fluid from adaptation system	Expt. IV Fructose	Expt. IV Glucose
(*a*) Initial suspension	16	5	—	15	15	—	45	49
(*b*) Incubated with raffinose for 3·5 hr.	182	164	166	192	140	—	140	148
(*c*) Cells from (*b*) recovered and:								
(i) resuspended in buffer without washing	—	—	—	71	—	—	—	—
(ii) washed twice with distilled water	33	48	146	—	51	101	130	139
(iii) washed twice with 0·07 M-KH_2PO_4	—	50	155	66	—	—	—	—
(iv) washed twice with 0·07 M-KH_2PO_4 + 0·01 M-cysteine	—	—	—	64	—	—	—	—

Table 9. *Invertase and raffinase content of* S. fragilis *grown in various media*

(Invertase and raffinase contents given as units/mg. dry wt. of cells.)

	Additions to basal medium: None	2% Raffinose		2% Galactose	2% Lactose	2% Glucose		10% Glucose	2% Fructose		10% Fructose		2% Sucrose	
Growth time (hr.)	16	24	48	16	16	16	42	16	16	48	16	48	24	48
(*a*) Invertase	157·7	138·0	123·8	33·5	29·6	0·7	21·2	0·54	0·56	31·8	0·24	0·83	0·48	23·2
(*b*) Raffinase	15·6	16·2	13·6	4·1	4·7	—	1·5	0·07	0·09	4·05	0·04	0·12	0·05	2·9
Ratio *a*/*b*	10·1	8·5	9·1	8·2	6·3	—	14·1	7·7	6·2	7·9	6·0	6·9	9·6	8·0

bating the cells with raffinose until adaptation was complete and then testing the ability of the cell-free suspension fluid to hydrolyse raffinose. There was no hydrolysis in 16 hr. The loss in activity was very much less if the adapted cells, washed twice with distilled water, were replaced in the fluid in which the adaptation was originally carried out, instead of in fresh raffinose solution. Moreover, the twice-washed cells could ferment fructose at an almost undiminished rate (Table 8, Expt. IV). If the maximum rate of carbon dioxide production from raffinose is only attained when an appreciable amount of fructose has accumulated in the medium (which may be a slow process), then the reduction in the rate of carbon dioxide production from raffinose brought about by washing the adapted cells could be due to the removal of fructose. Against this explanation, however, is the fact that the cells show no loss of activity on washing if they have been grown in basal medium plus 2% raffinose. For example, a sample of such a culture to which extra raffinose was added had a $Q_{CO_2}^{N_2} = 181$, while for the cells washed twice in distilled water $Q_{CO_2}^{N_2} = 164$. This difference in behaviour between cells adapted to raffinose in suspension and those adapted during growth could be easily explained if the latter cells had a much higher raffinase content than the former. This would allow a sufficient concentration of fructose to be built up much more quickly. However, direct measurement of the raffinase content of *S. fragilis* grown in basal medium, either alone or with the addition of 2% raffinose, shows no appreciable difference between the two, and in both cases it is much higher than is necessary to account for the observed value of $Q_{CO_2}^{N_2}$ (raffinose) for adapted cells (Table 9).

Raffinase content of cells grown under various conditions. A comparison of the raffinase and in-

Table 10. *Raffinase formation during adaptation to sucrose by washed-cell suspensions of* S. fragilis

(Experimental conditions as for Fig. 12, Expt. 1. Results given as invertase or raffinase units/mg. dry wt. of cells.)

	Initial enzyme	Sugar added to adaptation system			
		None	Sucrose	Glucose	Raffinose
(*a*) Invertase	0·54	0·44	7·85	0·68	0·63
(*b*) Raffinase	0·08	0·07	0·93	0·06	0·10
Ratio *a*/*b*	6·8	6·3	8·4	10·1	6·3

vertase contents of *S. fragilis* grown in various media is given in Table 9. It will be seen that there is only a twofold variation in the ratio (invertase content)/(raffinase content) for a 200-fold variation in the raffinase content. Fig. 8 shows that the invertase and raffinase activities vary in a similar manner during growth of the yeast on basal medium plus 1 % raffinose, the ratio of the two activities ranging from 5·8 to 9·8.

Raffinase content of cells following adaptation to sucrose. If raffinose and sucrose are hydrolysed by different enzymes then the two enzymes might be expected to respond differently when unadapted cells are induced to synthesize one or other enzyme in washed-cell suspensions. Table 10 shows that when unadapted *S. fragilis* is induced to form invertase by incubating in a sucrose-buffer mixture, raffinase is also formed. The difference in the ratios of the two activities before and after adaptation is probably not significant.

DISCUSSION

It is well known that the formation of many enzymes is suppressed when glucose is present in the growth medium (see review by Gale, 1943). Invertase formation by *S. fragilis* provides yet another example of this phenomenon. Not only is invertase formation in growing cultures suppressed, but its formation by washed cell suspensions, where cell multiplication is probably unimportant, is likewise prevented if a sufficiently high concentration of glucose is present. Fructose, so far as its effect has been examined, behaves similarly to glucose. If the enzymic activities exhibited by cells grown in basal medium alone are assumed to represent the levels attainable in the absence of any inhibition, then galactose, lactose and sucrose also inhibit the formation of invertase and raffinase. It is difficult to differentiate between the possibilities that the reduction in enzyme content is due to a true inhibition of the invertase and raffinase synthesizing systems by the sugars, or to an inability of these systems to compete with other systems for the available raw materials (cf. Monod, 1947; Spiegelman, 1946; Mandelstam, 1952; Mandelstam & Yudkin, 1952). The fact that sucrose is also apparently inhibitory is perhaps significant in this connexion. Hestrin & Lindegren (1952) have shown that the formation of maltase and of α-methylglucosidase is depressed when glucose is present in the growth medium along with maltose or α-methylglucoside, and they point out that 'the formation of glucosidases in a medium in which glucose is replaced by a glucoside may result simply from the absence of glucose and is not necessarily an outcome of an adaptive response to the glucoside as such'. They also suggest that in a system which has synthesized a glucosidase beyond the level necessary to keep the fermentation system maximally employed, the rising glucose concentration might prevent excessive formation of the glucosidase. No convincing evidence was presented in support of these ideas. The experiments described in this paper, however, do lend considerable support to these suggestions, at least for the enzyme invertase. When the effect of glucose is removed by growing the yeast in basal medium alone, the invertase content of the cells rises to about 10 times that found in cells grown in the presence of 2 % sucrose. Nevertheless, the invertase content of the sucrose-grown cells is still more than sufficient to account for the observed rates of fermentation of glucose and sucrose. The very high invertase content of cells grown in raffinose medium can also be readily explained if the assumption is made that raffinase and invertase are the same enzyme (see below). Raffinose is hydrolysed at only one-tenth the rate of sucrose, so that with raffinose as substrate the amount of enzyme needed to allow hexose to accumulate at the same rate as with sucrose as substrate would have to be about 20 times as great (raffinose hydrolysis yields only one fermentable hexose; sucrose yields two). If therefore accumulation of free hexose is in fact responsible for limiting the amount of invertase synthesized, the limit would not be reached with raffinose as substrate until 20 times as much invertase had been formed as with sucrose. Other factors probably come into play before this limit is reached and, as a result, cells grown in raffinose medium respond as if there was no hexose present and have the same invertase content as cells grown in basal medium alone.

It is a reasonable assumption that, in those cases of enzyme formation in which the inducing substrate is also the source of energy for enzyme synthesis, the

rate of formation of the enzyme will be a direct function of the initial amount of enzyme within the cells. On this basis failure of *S. fragilis* cells grown in glucose medium to adapt to ferment galactose, lactose and raffinose could be due to complete suppression, by glucose, of the formation of one or more of the enzymes required for the adapted state. Growth in the basal medium alone would allow a small synthesis of these enzymes, sufficient for adaptation to occur when the cells are incubated with the inducing substrate. It would appear from the effect of fructose on invertase formation in cell suspensions, and also from the invertase contents of cells grown with fructose, galactose or lactose as carbon sources, that inhibition of enzyme formation is not a specific property of glucose. Fructose would seem to be equally as effective as glucose. The reduced invertase content of cells grown in basal medium plus galactose or lactose, as compared with basal medium alone, could however be a competition effect.

With regard to the hydrolysis of raffinose by *S. fragilis*, the results presented in this paper show that despite a 200-fold variation in the ability of this yeast to hydrolyse sucrose and raffinose, the ratio of the two activities shows only a twofold variation. The values obtained for this ratio, 6·3–14·2, are not very different from those obtained by Adams *et al.* (1943), 4·11–8·59, for various preparations of purified invertase from brewer's and baker's yeasts. The observations do not support the view that sucrose and raffinose are hydrolysed by different enzymes in *S. fragilis*, but rather the reverse, that the same enzyme is involved in both cases.

It has been shown that *S. fragilis* cells grown in basal medium plus 10% glucose develop increased invertase and raffinase activities when incubated with sucrose at pH 4·5 for 2–3 hr., and at the same time the rate of sucrose fermentation rises to that for glucose. Despite the fact that the unadapted cells possess raffinase activity, they do not exhibit an increased ability to ferment raffinose when exposed to this sugar for as long as 6 hr., nor is there any change in their raffinase content. Failure to adapt in the presence of raffinose could be due to inability of this sugar to induce enzyme formation (cf. Monod, Cohen-Bazire & Cohn, 1951), but this seems unlikely. A more probable explanation would be that it is due to the much lower activity of the unadapted cells towards raffinose than towards sucrose. Invertase formation in the presence of sucrose shows a lag of about 30 min., and if the length of the lag is a function of the activity of the enzyme initially present in the cells, then a reduction in activity to one-tenth, as is the case when raffinose is substituted for sucrose, might be expected to lengthen the lag considerably. Even at the end of the lag period, the rate at which energy can be made available from raffinose is still only one-tenth of the rate from sucrose (one-twentieth if both hexoses from sucrose are completely fermented), which again would lengthen the adaptation time. The combination of these two effects may well result in no observable adaptation to raffinose within a period of 6 hr.

The raffinase activity of the yeast grown in basal medium alone is as high as that of yeast grown in the presence of raffinose. This would indicate that raffinase is not an adaptive enzyme in *S. fragilis*. Nevertheless, the manometric evidence shows that a period of adaptation is necessary before carbon dioxide is evolved from raffinose at the maximum rate and that azide prevents the attainment of this maximum rate. It would seem therefore that fermentation of raffinose by *S. fragilis* involves the formation of an adaptive enzyme, but that the enzyme concerned is not the one responsible for the hydrolysis of raffinose. The rate of fermentation of glucose and of fructose by cells grown in basal medium alone is also very low initially and increases slowly during contact with the substrate, the increase being prevented by azide. This would suggest that increases in some of the enzymes of the glycolytic system may be a factor in glucose fermentation by such cells and, consequently, in raffinose fermentation also. Fowler (1951) has suggested that the enzyme system responsible for anaerobic utilization of glucose by *Escherichia coli* can behave as an adaptive system.

SUMMARY

1. The ability of washed cell suspensions of *Saccharomyces fragilis* to ferment glucose, sucrose, galactose, lactose and raffinose following growth in a basal medium, either unsupplemented or supplemented with one of these sugars, has been studied.

2. Ability to ferment galactose, lactose or raffinose is an adaptive property in *S. fragilis*.

3. Following growth in basal medium plus 10% glucose, washed-cell suspensions of *S. fragilis* do not acquire the ability to ferment galactose, lactose or raffinose when incubated with these sugars in phosphate buffer, pH 5·0, for 6 hr. The initial ability of such cell suspensions to ferment sucrose is very low, but when incubated with sucrose and phosphate buffer for 2 hr. the fermentation rate increases to that for glucose fermentation. This increase is prevented by 3×10^{-4} M-sodium azide.

4. The increase in the rate of sucrose fermentation is accompanied by an increase in invertase activity, sufficient to account for the increase in fermentation rate. The increase in invertase activity is most rapid with very young cells and at pH 4·5–5·0, and does not occur in presence of sodium azide.

5. Following growth in unsupplemented basal medium, washed-cell suspensions of *S. fragilis* can acquire the ability to ferment raffinose, lactose and galactose, the rate of adaptation decreasing in that order. Adaptation does not occur in presence of sodium azide. The initial rate of glucose and fructose fermentation by these suspensions is very low and maximum rate is not reached for 2 hr., the increase in rate being prevented by sodium azide.

6. Failure to ferment raffinose following growth in unsupplemented basal medium is not due to lack of hydrolytic activity towards raffinose, but appears to be due to a deficiency in the glycolytic system.

7. Evidence is presented which suggests that glucose and fructose strongly inhibit invertase synthesis by *S. fragilis*.

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Fibrinolysins of Human Plasma

A COMPARISON OF FIBRINOLYTIC PLASMA FROM NORMAL SUBJECTS AND FROM CADAVER BLOOD WITH PLASMIN PREPARED BY ACTIVATION WITH CHLOROFORM

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This paper is concerned with the nature of the fibrinolytic activity which develops in human blood in a variety of circumstances. The centre of interest is the spontaneous fibrinolysis produced in the normal subject by physical or emotional disturbances such as severe exercise or fear or by injection of adrenaline (Macfarlane, 1937; Macfarlane & Pilling, 1946; Biggs, Macfarlane & Pilling, 1947). Fibrinolytic activity can also be produced *in vitro* by treating plasma with chloroform or certain bacterial filtrates, and this has been shown to be due to the conversion of a zymogen ('plasminogen') present in normal plasma to its active, proteolytic form ('plasmin') (Christensen, 1945, 1946; Christensen & MacLeod, 1945). Normal plasma and serum were found to contain a powerful inhibitor to plasmin. Intense fibrinolytic activity sometimes occurs in blood taken post mortem, particularly in cases of sudden or violent death (Mole, 1948).

The question arises whether these phenomena are due to the same active agent. Macfarlane & Pilling (1946) considered that spontaneous fibrinolysis in living subjects was due to activation of the enzyme plasmin characterized by Christensen, and to a decrease in antiplasmin. Mole (1948) pointed out differences between post-mortem fibrinolysin ('cadaver lysin') and plasmin, and Bidwell & Macfarlane (1951) found that the fibrinolytic activity produced in living subjects by exercise also differed from plasmin, so that further identification of the factors concerned is clearly required.

Recent work has emphasized the lability of plasma fibrinolysin (Fearnley, Revill & Tweed, 1952; Truelove, 1953; Fearnley & Tweed, 1953), a finding which may explain discrepancies in previous observations. Using the test devised by Macfarlane (1937) and modified by Macfarlane & Biggs (1946), it was shown that, if the interval between collection of the specimen and setting up the test is sufficiently brief (about 15 min.), most normal subjects give a positive result; so that fibrinolysis observed after exercise, and even in cadaver blood (Mole, 1948), may arise from a quantitative rather than a qualitative change in the state of the blood.

The following paper presents some further observations on the inter-relationship of the fibrinolytic phenomena produced respectively by exercise, by sudden death and by the action of chloroform, and on the technical difficulties of testing. Some of the results have been the subject of a preliminary communication (Bidwell & Macfarlane, 1951).

METHODS

Materials

Plasma. Citrated human plasma, used as the source of fibrinogen, thrombin and plasmin, was obtained from the hospital 'blood bank'.

Fibrinogen. The phosphate precipitation method of Jaques (1943) was modified as described by Biggs & Macfarlane (1953). The dried material contained salts (about 60%, w/v); the protein was about 85% clottable; it was contaminated with plasminogen (i.e. clots were lysed by streptokinase).

Thrombin. Solutions of the dried material (prepared as described by Biggs & Macfarlane, 1953) were made fresh for each experiment in a concentration of 20 units/ml. (using Thrombin Topical, Parke, Davis and Co., as standard).

Fibrinolysin preparations. '*Exercise plasma.*' The exercise entailed running up and down a spiral, stone staircase, 39 ft. in vertical height, some 10–14 times until the subject felt exhausted. Blood was taken by venepuncture and mixed with one-ninth its volume of 3·8% (w/v) trisodium citrate; centrifuged for 10 min. at 2000 rev./min., and the plasma separated. In a few experiments as specified the anticoagulant was NaCl.

'*Normal plasma.*' This was prepared in the same way from blood withdrawn from normal subjects before exercise.

'*Cadaver lysin.*' When blood was found to be fluid at routine post-mortem examinations (see Mole, 1948) as much as possible was withdrawn from the femoral vein. In the majority of cases the blood remained fluid after collection without anticoagulant and could not be clotted by thrombin. Since therefore fibrinogen was shown to be absent it could be concluded that fibrinolysis had taken place in the vessels before collection of the sample. The fluid obtained was thus a mixture of blood cells, serum and the products of fibrinolysis; the cells were removed by centrifuging. In two cases the blood clotted after collection; in these samples no fibrinolytic activity could be demonstrated. It was found that the fibrinolytic activity of cadaver blood was very much greater than that of specimens from living subjects but the volume of blood obtainable after death was usually very small.

Plasmin. Chloroform-activated plasmin was prepared by the method of Rocha e Silva & Rimington (1948). It was found that such preparations would clot plasma, but that equally active preparations which did not clot plasma could be made if the starting material was first treated with alumina as described for fibrinogen by Biggs & Macfarlane (1953). The clotting properties appeared to be due to contamination by thrombin and not to be a function of plasmin (Nikolic, 1951).

Determination of comparative amounts of fibrin in partially lysed and whole clots

Reagents. All reagents were of analytical quality where available. *Buffered saline.* 5·0 ml. sodium diethylbarbiturate:sodium acetate buffer base (Michaelis, 1931)+5·0 ml. 0·1 N-HCl+90 ml. 0·85% (w/v) NaCl (pH 7·4 measured with a glass electrode). This buffer was used for making up fibrinogen solutions and as a diluent. *Sodium hydroxide.* 0·1 N-NaOH, filtered before use. *Sodium carbonate.* 12·5% (w/v) Na_2CO_3, filtered before use. *Copper sulphate.* 0·01 M-$CuSO_4$. *Phenol reagent.* Folin & Ciocalteu (1927) phenol reagent (from British Drug Houses Ltd.). One vol. of the stock reagent was diluted with two vol. of distilled water just before use.

Determination of fibrin. Clots were collected by winding on to a glass rod, roughened over the lower part, which had been left *in situ* after use as a stirring rod for mixing the reagents before clotting. The rod, with the adhering clot, was transferred to another tube containing about 10 ml. of ice-cold 0·85% (w/v) NaCl in an ice-water bath. The tubes of this second series had a capacity of about 12 ml. and were graduated at 8·0 ml. The clot, still attached to the rod, was washed with two more changes of ice-cold saline (about 30 ml. in all) and then dissolved by adding 2·0 ml. NaOH solution down the rod and immersing the tube in a boiling-water bath for 2–3 min. After cooling, the volume was made up to 8·0 ml. with Na_2CO_3 solution. The mixture could then be left until it was convenient to develop the colour.

$CuSO_4$ solution (1·0 ml.) was added and mixed, followed by 1·0 ml. of diluted phenol reagent (added from a glass syringe with rapid stirring). The colours were read after not less than 0·5 hr. using a photoelectric colorimeter and an Ilford red filter; the blank was prepared from the reagents. The colour was recorded in terms of the amount of tyrosine required to produce the same colour under the same experimental conditions.

It was found that this procedure is preferable to other modifications of the Folin & Ciocalteu method; it is well recognized that the colour is due to other factors in addition to tyrosine.

Measurement of plasmin activity

(1) *The method of Rocha e Silva & Rimington* (1948). 0·5 ml. enzyme or enzyme-inhibitor mixture + 0·30 ml. fibrinogen (1·3 mg. dried preparation/ml.) + 1 drop thrombin, incubated at 37°. The lysis time was recorded when the bubbles in the clot rose to the surface. One unit of enzyme activity was the amount required to effect lysis in 10 min. This method was used in following the stages of purification of plasmin and for a few experiments with cadaver lysin; it was insufficiently sensitive for work with exercise plasma. It has the theoretical disadvantage of requiring complete destruction of the substrate.

(2) *The method of partial lysis.* The method was much more sensitive than (1) and is theoretically more sound, but in practice is laborious. A solution of fibrinogen was prepared of such concentration that when 4·0 ml. of fibrinogen solution + 1·0 ml. buffered saline was clotted with 0·2 ml. thrombin, the amount of clot, as determined by the method given above, was equivalent to 144 μg. tyrosine. In the determination of enzyme activity, a mixture of 4·0 ml. fibrinogen solution + 1·0 ml. enzyme (or enzyme-inhibitor mixture prepared previously) was placed in a water bath at 37°. After 5 min. incubation, to allow the mixtures to reach 37°, 0·2 ml. thrombin was added and thoroughly and rapidly mixed using a glass rod roughened over the lower part. The rod was left in the tube. Exactly 45 min. after the addition of thrombin the amount of clot remaining was determined by the method given above for the determination of fibrin. One unit of enzyme by this method was defined as the amount required to lyse 25% of the clot in 45 min. at 37°, the amount of fibrinogen and thrombin and the final volume being fixed as described (approx. 2·5 units by this method = 1 unit by method (1)).

Demonstration of fibrinolysis in exercise plasma

'*Dilution test*' (Macfarlane, 1937; Macfarlane & Biggs, 1946). 0·4 ml. of exercise plasma was added to 6·0 ml. of buffered saline to give a dilution of 1/16. Two sets of three tubes were prepared as follows: (*a*) 1·6 ml. diluted plasma. (*b*) 0·8 ml. diluted plasma + 0·8 ml. buffered saline. (*c*) 0·4 ml. diluted plasma + 1·2 ml. buffered saline. To each tube of one set was added one drop of thrombin and to each of the second set 0·1 ml. of 0·05 M-$CaCl_2$. The contents of the tubes were mixed by inverting. The six tubes were placed in a water bath at 37°, together with a similar set of tubes prepared from normal plasma. The test should be made at a standard time after withdrawing the blood because the fibrinolytic effect may disappear during storage. The tubes were observed to ensure that coagulation occurred and examined again after 24 hr. for lysis of the clot. When possible the tubes were observed more frequently as the lysis time gives an approximate measure of the fibrinolytic activity.

Method of partial lysis. This method is similar to the method of partial lysis described for plasmin. The differences are (*a*) the source of fibrinogen in the plasma itself, (*b*) the incubation times are necessarily much longer. Buffered saline (4·8 ml.) was placed in a series of tubes; 0·2 ml. exercise plasma was added, mixed with a rod and placed in a water bath at 37°. The mixtures were allowed 5 min. to reach 37° before starting the experiments. Thrombin (0·2 ml.) was added at this time unless otherwise stated. Clots were collected after the desired incubation time and the amount of fibrin determined as described above.

Demonstration of fibrinolysis by cadaver lysin

The techniques for plasmin and exercise plasma can be modified to show the high fibrinolytic activity of cadaver lysin. Fibrinogen is not present in cadaver lysin and must be added either in the form of a solution of purified fibrinogen or as normal plasma. Mole (1948) used plasma from the hospital blood bank; we have used 0·2 ml. normal plasma or 0·3 ml. 'bank' plasma in a final volume of 5·0 ml.

RESULTS

Difference between 'thrombin clots' and 'calcium clots'

In the preliminary work on exercise plasma it was necessary to decide whether to clot the citrated plasma by the addition of thrombin or by recalcification. Fig. 1 shows that the speed of lysis when

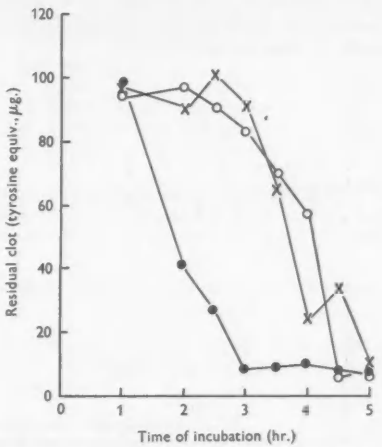

Fig. 1. The difference between 'thrombin clots' and 'calcium clots'. Each tube contained 0·2 ml. exercise plasma and 5·5 ml. buffered saline. Clotting produced by: 0·2 ml. 1·1% (w/v) $CaCl_2$, ×—×; 0·2 ml. 1·1% (w/v) $CaCl_2$ + 0·2 ml. thrombin, ○—○; 0·2 ml. thrombin, ●—●. Mixtures were incubated in water bath at 37°. Similar sets of clots from plasma taken before exercise had given no evidence of lysis at the end of the experiment.

calcium ions and thrombin were both added to the plasma was the same as for calcium ions alone and was slower than that of the clots formed with thrombin alone in the absence of added calcium. The effect therefore cannot be due to plasmin in the thrombin nor to activation of the intrinsic clotting system of the plasma.

It was possible that such a result might arise from activation by excess citrate. The experiment was repeated using sodium chloride as an anticoagulant (Richardson, 1858) instead of sodium citrate.

Blood obtained by venepuncture was mixed with an equal volume of 10 % (w/v) sodium chloride. It was diluted for use with 9 vol. of (*a*) 0·167 %, w/v, sodium chloride; (*b*) 0·167 (w/v) sodium chloride containing 0·0005 M-calcium chloride; (*c*) 0·167 % (w/v) sodium chloride containing 0·0025 M-calcium chloride. The mixtures of series (*a*) were clotted with thrombin; those of series (*b*) and (*c*) were allowed to clot naturally. The final concentration of calcium in mixture (*b*) was approximately that of ionized calcium and in mixture (*c*) of total calcium in plasma. The final concentration of sodium chloride was physiological and the mixtures contained no foreign ions. The lysis of the clots followed the same course as in Fig. 1, namely series (*b*) and (*c*) lysed at the same rate, despite a fivefold difference in added calcium, and series (*a*) lysed more rapidly. The difference is therefore not due to citrate nor probably to direct inhibition by calcium ions. It was decided that subsequent experimental work would be more conveniently done using thrombin.

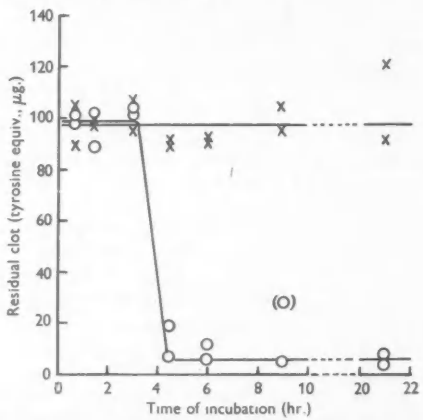

Fig. 2. Time lag in lysis of clot in exercise plasma. Plasma before exercise, ×—×; plasma after exercise, O—O.

Time lag in digestion of clots in exercise plasma

The course of lysis of the clots in Fig. 1 suggested that there was not a steady decrease in the amount of clot collected but rather that the amount remained constant for a certain time and then began to diminish rapidly. This was confirmed in the experiment shown in Fig. 2 in which no decrease in the amount of clot was demonstrable during the first 3 hr. of incubation, yet within the next 1½ hr. all the clot had been digested.

Inhibition of plasmin by exercise plasma

A suggestion that fibrinolytic plasma obtained after exercise might contain less plasmin inhibitor than normal plasma (Macfarlane & Pilling, 1946) was tested with the result shown in Fig. 3. It was found to be inhibitory to the same extent as normal plasma in the reaction between plasmin and fibrin. It was also found that the plasmin inhibitor of plasma was effective in this system only if the mixture of enzyme and inhibitor was made first and afterwards added to the substrate (fibrinogen).

It seemed therefore that the other suggestion of Macfarlane & Pilling (1946), namely that fibrinolytic plasmas contain an increased amount of plasmin, might still be true, since this plasmin would be formed in the presence of the substrate, fibrinogen, and therefore might be unaffected by plasmin inhibitor. This supposition was tested in two ways (i) by the preparation of 'artificial fibrinolytic plasma' (a mixture of plasmin and normal plasma),

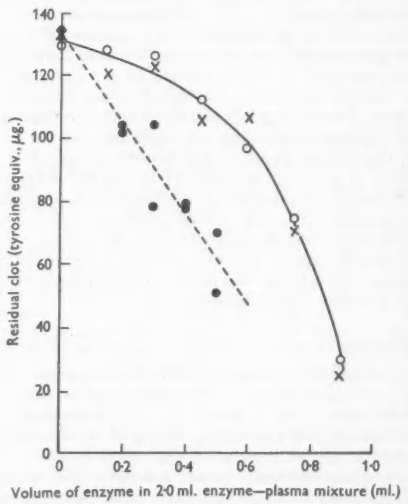

Fig. 3. Inhibition of plasmin by plasma samples taken before and after exercise. Two ml. enzyme-inhibitor mixture contained: 0·004 ml. plasma before exercise, ×—×; 0·004 ml. plasma after exercise, O—O; no inhibitor, ●—●. Mixtures stood 0·5 hr. at room temperature, 1·0 ml. added to 4·0 ml. fibrinogen at 37°. After 5 min., thrombin (0·2 ml.) was added. Clots were collected after 45 min. at 37° and estimated by the standard method.

and (ii) by investigating the properties of mixtures of fibrinolytic and normal plasma.

(i) In attempts to prepare artificial fibrinolytic plasma by adding plasmin to normal plasma it was found that the addition of a relatively large amount of plasmin might produce a mixture which would lyse rapidly, e.g. in 15–20 min., while if only a little less plasmin were used the clot would not lyse at all (see Table 1). In these experiments enzyme and

inhibitor were 100 times as concentrated as when purified fibrinogen was used as the substrate (see Fig. 3) and the plasmin inhibitor appeared to be effective in the presence of fibrinogen.

Table 1. *Lysis times of plasmin-plasma mixtures*

(Each mixture contained 0·2 ml. plasma and varying amounts of plasmin added to sufficient buffered saline to bring the volume to 5·0 ml. The mixtures were clotted with thrombin and incubated at 37°.)

Plasmin (ml.)	Lysis time
0·8	No proper clot formed
0·7	7–12 min.
0·6	13 min.
0·5	>15 <35 min.
0·4	No lysis after 24 hr.
0·3	No lysis after 24 hr.
0	No lysis after 24 hr.

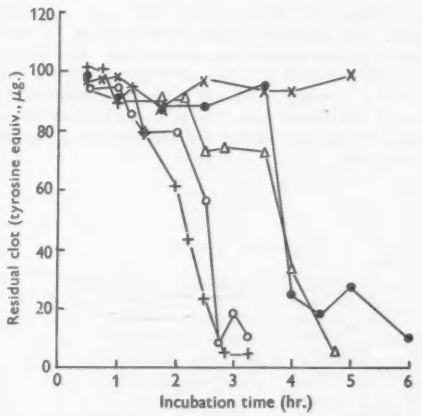

Fig. 4. Effect of mixing exercise plasma and normal plasma. Blood was taken into equal volume of 10% (w/v) NaCl, before and after exercise. Diluent: 1 vol. Michaelis buffer base + 1 vol. 0·1 N-HCl + 8 vol. distilled water. Plasma (0·5 ml.) + 4·5 ml. diluent placed in water bath at 37°. Thrombin (0·2 ml.) was added after 5 min. Clots were collected after incubation times as shown (abscissa).

Mixtures of plasma		
Before exercise (%)	After exercise (%)	
0	100	+—+
20	80	○—○
40	60	△—△
55	45	●—●
100	0	×—×

The results in Table 1 are in marked contrast to those shown for exercise plasma in Fig. 2, and it was impossible to imitate this type of lysis; another point of difference was that the artificial lytic clots fragmented so that it was impossible to collect them quantitatively on to stirring rods as was done with the fibrinolytic clots made from exercise plasma.

(ii) The activity of mixtures of normal and exercise plasmas was found to depend only on the proportion of exercise plasma in the mixture (Fig. 4). This difference between exercise plasma and an artificial mixture of plasmin and plasma was confirmed by preparing such an artificial fibrinolytic plasma and using it in mixtures with normal plasma in exactly the same way as in the previous experiment. Even a small admixture of normal plasma was sufficient to prevent fibrinolysis.

Table 2. *Lysis of plasma clots by dilutions of cadaver lysin*

(Each mixture contained 0·3 ml. 'bank' plasma and varying amounts of lysin added to sufficient buffered saline to bring the final volume to 5·0 ml. The mixtures were clotted with thrombin and incubated at 37°.)

Cadaver lysin (ml.)	Lysis time
0·15	<30 min.*
0·075	48 min.
0·0375	75 min.
0	No lysis overnight

* After lysis had occurred a second amount of 'bank' plasma (0·30 ml.) was added to this tube (thereby doubling the plasmin inhibitor); lysis again took place in 20–30 min.

Table 3. *Inhibition of plasmin by fibrinolytic cadaver 'serum'*

(Method of measurement of activity as for plasmin by method (1). 0·25 ml. Plasmin was mixed with 0·25 ml. inhibitor in (*a*) and (*b*).)

Inhibitor	Lysis time min.	sec.
(*a*) Fresh normal plasma		
1/50	60	0
1/100	14	15
1/200	11	0
Physiological saline	5	35
(*b*) Cadaver lysin		
1/12·5	18	0
1/25	17	0
1/50	11	55
1/100	7	35
1/200	6	55
Physiological saline	5	25
(*c*) 0·25 ml. physiological saline + 0·25 ml. cadaver lysin 1/12·5	16–19	

Cadaver lysin behaves in this respect like exercise plasma, since dilutions of lysin in mixtures containing a constant amount of plasma seem to be unaffected by any inhibitor in the plasma (see Table 2 and contrast with Table 1). Crude cadaver lysin itself contains an inhibitor to plasmin (Table 3).

Comparison of the fibrinolytic activity of cadaver lysin and plasmin tested against clots made from (a) crude plasma, (b) purified fibrinogen

Exercise plasma could not be used in this experiment because it is insufficiently active. The results in Table 4 show that the cadaver lysin was much less active than the plasmin in dissolving a clot made from purified fibrinogen but more active in dissolving a clot made from crude plasma.

Table 4. *Comparison of the fibrinolytic activity of cadaver lysin and plasmin tested against clots made from (a) crude plasma, (b) purified fibrinogen*

((*a*) A modification of the method of Mole (1948) described in Table 2. (*b*) Method as for plasmin by method (1).)

Method	Enzyme (ml.)	Lysis times: Cadaver lysin min.	sec.	Plasmin (10 mg./ml.) min.	sec.
(*a*) (Crude plasma)	0·2	45	0	No lysis	
	0·1	75	0	No lysis	
	0·05	100	0	No lysis	
(*b*) (Purified fibrinogen)	0·125	17	0	2	30
	0·0625	14	0	3	55
	0·0313	15	30	6	30
	0·0156	19	0	13	30

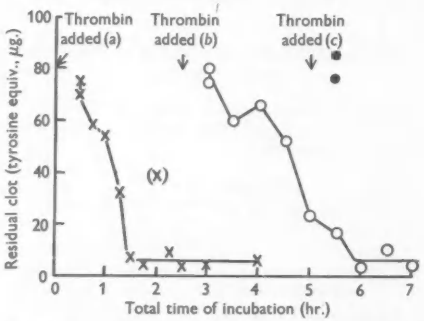

Fig. 5. The effect of exercise plasma on fibrinogen. Blood was taken before and after exercise into an equal volume of 10% (w/v) NaCl. Plasma (0·50 ml.) + 4·50 ml. 0·167% NaCl in water bath at 37°. Zero time for reaction 5 min. after placing in bath. Thrombin (0·2 ml.) was added at times indicated on graph (*a*) immediately, ×—×; (*b*) after 2½ hr., ○—○; (*c*) after 5 hr., ●—●. In the controls from plasma taken before exercise the clots were still intact next day.

The effect of exercise and cadaver fibrinolysins on fibrinogen

Fig. 5 shows that fibrinogen did not appear to be attacked at all in exercise plasma; the same result was obtained with cadaver lysin (Fig. 6). These experiments were made with plasma clots; Fig. 7

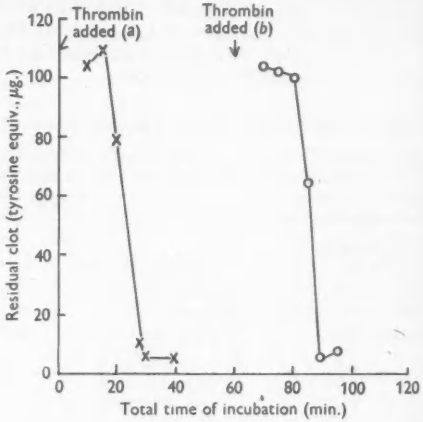

Fig. 6. Effect of cadaver lysin on fibrinogen in plasma. Each tube contained 4·5 ml. buffered saline, 0·30 ml. 'bank' plasma and 0·20 ml. cadaver lysin. Zero time for reaction was taken as 5 min. after placing in water bath at 37°. Thrombin (0·2 ml.) added (*a*) immediately, ×—×; (*b*) after 60 min., ○—○. In the controls without lysin, the amount of clot did not diminish during the experiment.

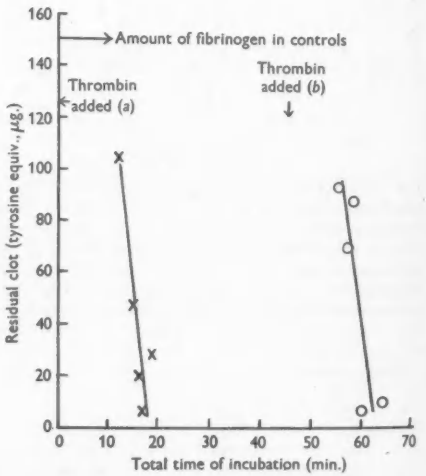
Amount of fibrinogen in controls
Thrombin added (a)
Thrombin added (b)
Residual clot (tyrosine equiv., μg.)
Total time of incubation (min.)

Fig. 7. Effect of cadaver lysin on purified fibrinogen. Each tube contained 4·0 ml. fibrinogen solution and 1·0 ml. of cadaver lysin diluted 1 in 12·5. Zero time for reaction was 5 min. after placing in water bath at 37°. Thrombin added (*a*) immediately, ×—×; (*b*) after 45 min., ○—○. In the controls without lysin the amount of clot did not diminish during the experiment.

shows the action of a very active cadaver lysin on fibrin made from purified fibrinogen. The clots were lysed completely 15–20 min. after adding the thrombin, but the amount of clot obtained and the time required for lysis were the same after a preliminary incubation of the mixture of fibrinogen and lysin for 45 min. before adding thrombin.

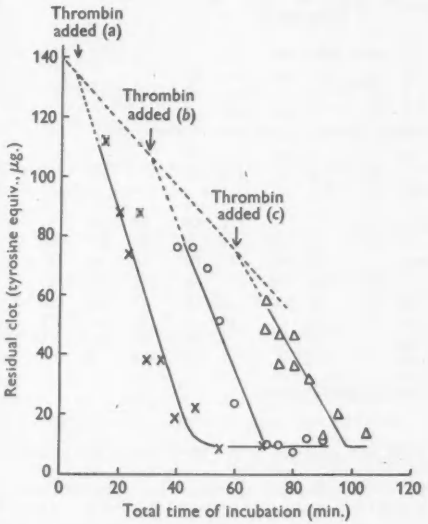

Fig. 8. Comparison of rates of fibrinolysis and fibrinogenolysis by plasmin. Each tube contained 4·5 ml. fibrinogen solution, and 0·5 ml. plasmin, and was placed in a water bath at 37°. Thrombin was added (*a*) after 5 min., ×—×; (*b*) after 30 min., ○—○; (*c*) after 60 min., △—△. Extrapolation of each curve through the values obtained for fibrin to the time of the corresponding addition of thrombin gave an estimate of the amount of unchanged fibrinogen present at that moment and enabled the rate of fibrinogenolysis to be determined. The amount of clot recovered from the control mixtures (without plasmin) did not diminish during the experiment.

In view of these results the action of plasmin on fibrinogen and fibrin was re-investigated by the method of partial lysis. The rate of destruction of fibrin was found to be approximately 2·5 times as fast as the rate of destruction of fibrinogen (see Fig. 8).

Comparison of the properties of cadaver lysin and plasmin

pH *optimum*. The method of determination of enzymic activity was that of Rocha e Silva & Rimington (1948) as described above for plasmin (method 1); it was chosen since the whole determination is completed in about 15 min., thus minimizing the effects of variation in enzyme stability at different pH values (Table 5).

Neither plasmin nor cadaver lysin show a well defined optimum; the most that can be said is that, over the range of pH values 6·5–8·5, plasmin is more active at the acid end of the range and cadaver lysin at the alkaline end. In view of the greater ease in observing the clots in the range pH 7·0–7·4, the pH used in all other determinations of activity was 7·2–7·4.

pH *of optimum stability*. Plasmin was heated to 37° for 30 min. in veronal:acetate:HCl buffers (Michaelis, 1931) at varying pH. After neutralization the solutions were tested by the method of Rocha e Silva & Rimington (1948). It was found that plasmin was most stable over the range pH 7·0–8·0 in good accord with Christensen & MacLeod (1945) who found pH 7·0–7·4. The amounts of cadaver lysin available were sufficient for only a much smaller experiment and attention was therefore concentrated on Mole's (1948) statement that cadaver lysin had the following properties: 'at pH 3–4 little activity was lost in 1 min. at 90°, while at pH 7·4 there was partial loss of activity at 60° for 10 min. and complete inactivation at 70°.'

The comparative stability of cadaver lysin at acid pH values was not confirmed. The results in Table 6 indicate that the loss in activity of cadaver lysin, crude or partially purified, was probably at least as great as that of plasmin (about 90 %).

As the operation of heating to 90° for 1 min. is so difficult to control, heating in a water bath at 45° for 30 min. was tried. Since the results in the previous experiment had differed from those of Mole (1948), it was considered that the test for activity should be the same as that used by Mole, namely lysis of plasma clots. The same conclusion was drawn from this experiment (see Table 7) as the previous one, namely that cadaver lysin was much less stable at pH 3–4 than at pH 7·0.

Fractionation of cadaver lysin. Mole (1948) stated that cadaver lysin differed from plasmin in that it was not found in the euglobulin fraction. The present experiments have not supported this statement since good recovery was found after either dilution with 20 vol. of distilled water and saturation with carbon dioxide or precipitation with ammonium sulphate at pH 7·0 (33 or 50 % saturation).

Is fibrinolytic activity due to a kinase?

Since Astrup has described a fibrinokinase present in tissue (Astrup & Permin, 1947; Astrup, 1951; Astrup & Stage, 1952), the effect of the presence of haemolysed red cells in a fibrin clot was investigated with the idea in mind that haemolysis might liberate a kinase. The result was completely

Table 5. pH *of optimum activity of plasmin and cadaver lysin*

(Buffer: sodium veronal:acetate:HCl (Michaelis, 1931). Fibrinogen (5·2 mg./ml.) in saline; samples were diluted with 3 vol. of the appropriate buffer and the pH values determined by glass electrode. The enzyme was diluted in the same buffer. The enzymic activity was determined by the method of Rocha e Silva & Rimington (1948) as described for plasmin method 1).)

			Dilution of enzyme											
			2		4		8		16		32		64		128	
			Lysis times											
Enzyme	Expt. no.	pH	min.	sec.	min.	sec.	min.	sec.	min.	sec.	min.	sec.	min.	sec.	min.	sec.
Plasmin	1	6·8	1	30	2	10	3	45	7	05	14	10	—		—	
		7·3	1	30	2	15	3	55	6	55	13	15	—		—	
		8·0	1	25	2	30	3	35	6	45	13	10	—		—	
		8·9	Clots all difficult to see and lysis times could not be determined											
	2	6·6*	—		1	5	3	5	7	20	17	30	—		—	
		7·3	—		2	40	4	20	9	50	19	50	—		—	
		8·4	2	20	3	15	5	25	11	15	—		—		—	
Cadaver lysin	1	6·6	—		No clot at 12 min.						(11	35)	15	35	25	0
		7·1	—		(7	0)	5	10	6	5	9	5	14	50	21	30
		8·3	—		No clot seen				(5	20)	12	10	10	5	15	0
	2	6·7	—		(11	10)	9	10	10	5	13	25	18	30	30	0
		7·0	—		8	50	9	35	8	55	10	45	17	50	24	0
		7·2	—		8	30	6	25	7	5	10	0	13	10	21	45
		7·7	—		No clot at 5 min.				7	5	8	45	12	50	21	45
		8·0	—		No clot at 5 min.				7–8	—	17	0	(13	0)	21	0

* At lower pH values the clot was unsatisfactory.

Table 6. *Effect of heating acidified solutions of cadaver lysin and plasmin to* 90° *for* 1 *min.* (*Mole*, 1948)

(The enzyme preparations used were (i) cadaver lysin, (ii) the euglobulin fraction of cadaver lysin and (iii) plasmin. Glass tubes 6 cm. long, 1·5 cm. diameter, walls 1 mm. thick, were used; 2·0 ml. solution in these tubes reached a temperature of 83° after plunging into a water bath at 90° for 1 min. 2 ml. amounts of enzyme solution were adjusted to pH 3–4 (glass electrode) by the addition of 0·1 N-HCl, heated as described above, cooled rapidly and the pH values readjusted to 7·2–7·4. Volumes were adjusted to 4·0 ml. and the enzymic activities determined by the method of Rocha e Silva & Rimington (1948) as described for plasmin (method 1).)

		Lysis times	
Enzyme	Dilution	Acidified and heated solutions	Unheated controls (min.)
Cadaver lysin	1	No lysis after 1 hr.	32–35
	2		Approx. 25
	4		Approx. 25
Euglobulin fraction of cadaver lysin	1		7–10
	2		9–11
	4		12–14
		min.	min. sec.
Plasmin	1	19	—
	2	24	2 5
	4	(30)	3 25
	8	(60)	6 35
	16	—	13 30

negative. The presence or absence of fresh platelets had no influence on the lysis time of plasma clots by cadaver lysin. Attempts were made to prepare artificial fibrinolytic plasma by adding streptokinase to fresh normal plasma, but the large variation in antistreptokinase in different specimens made the method impracticable. In the course of these experiments it was found that small amounts of streptokinase appear to be without effect on fibrinogen until clotting has taken place, while large amounts of streptokinase could activate the plasminogen before clotting. These observations were made possible by the use of refined concentrated antistreptococcal serum.

Table 7. *Effect of heating acidified cadaver lysins*

(The treated and control solutions were tested by the method of Mole (1948) as described in Table 2.)

Treatment	Volume of lysin used in test (ml.)	Lysis time
Lysin (2·0 ml.) acidified to pH 3–4, heated at 45° for 30 min. and neutralized. Vol. adjusted to 6·0 ml.	0·6	>3·75 <6 hr.
Lysin (2·0 ml.) at own pH heated to 45° for 30 min. and neutralized. Vol. adjusted to 6·0 ml.	0·6	70 min.
Lysin control, untreated	0·2	65 min.
Saline control	—	No lysis (24 hr.)

DISCUSSION

Difference between 'calcium clots' and 'thrombin clots'

Macfarlane & Biggs (1946) and Bidwell & Macfarlane (1951) reported that plasma clotted by calcium ions lysed more slowly than plasma clotted by thrombin. It has been shown that this difference is not due to contamination of the thrombin by plasmin, nor, probably, to direct inhibition by calcium ions. Fearnley & Tweed (1953), who have also remarked on this difference, attributed it to the interaction of calcium and 'another factor'. Lorand (1948, 1950, 1951) has provided good evidence for his suggestion that plasma clots made in the presence of calcium contain bonds which are not found in plasma clots made in the absence of calcium; such bonds would explain the greater resistance of 'plasma calcium clots' to fibrinolysis, and may provide the second factor suggested by Fearnley and Tweed. The fibrinolytic activity of purified thrombin described by Guest & Ware (1950) was manifested only by such highly concentrated thrombin (of the order of 10000 units/ml.) that it is improbable that it could have had any effect in these experiments.

Difficulties in measuring the degree of fibrinolytic activity

Fearnley and co-workers (1952, 1953) and Truelove (1953) have commented on the lability of the fibrinolytic phenomenon in plasma. Their method of assessing the degree of fibrinolytic activity is based on the work of Macfarlane (1937) and Macfarlane & Biggs (1946), and depends on the number of mixtures which lyse when a series of plasma dilutions are clotted by thrombin or calcium. Thus the mixtures differ not only in concentration of the active agent but also in all other constituents of the plasma, including the substrate and possible inhibitors. It is desirable to take a fixed dilution of plasma so as to control these factors. Unfortunately the most obvious method of determining enzymic activity, i.e. the determination of the amount of substrate changed after a given time, is precluded by the observation that digestion of clot is preceded by a variable time lag (Fig. 2). The total lysis time is probably some inverse function of the fibrinolytic activity and as yet provides the best method of measurement despite the disadvantage of requiring complete solution of the substrate. At present it appears that any method of comparing activity in specimens from different individuals can be only approximate as there will undoubtedly be differences in substrate concentration and possibly other relevant factors influencing lysis time.

Comparison of fibrinolysis by plasmin, exercise plasma and cadaver lysin

Normal plasma did not inhibit fibrinolysis by natural or cadaver lysin but was strongly inhibitory to plasmin. The ratios of activities of given quantities of cadaver lysin and plasmin on fibrinogen and plasma clots were different. Several authors have stated that plasmin attacks fibrinogen as readily as it attacks fibrin (e.g. Christensen, 1945; Permin, 1949; Westphal, Kocholaty, McDonald, King & Jensen, 1950), although Fantl & Simon (1948), investigating fibrinolysis following electrically induced convulsions, found that the fibrinogen was apparently unattacked. Experiments described above have shown that natural lysin and cadaver lysin fail to digest fibrinogen, while plasmin digests fibrinogen although at a slower rate than it digests fibrin. These results are not in conflict with those of Christensen (1946). He found identical rates of action of plasmin on fibrinogen and lysed clot, while here it is the rates of action of fibrinogen and of unlysed fibrin which are compared.

Mole (1948) found that cadaver lysin differed from plasmin as described in the literature by Christensen (1945, 1946) and others. Some of the properties of plasmin and cadaver lysin have now been compared under the same experimental conditions. Although confirmation has not been obtained of some of the most striking differences between cadaver lysin and plasmin reported by Mole (1948), his conclusion that the two are different entities is probably correct.

It appeared from the experiments reported above that the fibrinolysin produced *in vivo* is probably the same as that in post-mortem blood, although the activity is very much greater in the latter; neither system behaves as if the effect were due to an excess of plasmin preformed in the plasma, although the possibility that the actual lysis might be due to plasmin produced by an activator during or following clotting is not excluded.

Attempts to establish the existence of a kinase in fibrinolytic plasma have as yet been inconclusive. It may be relevant to note that under certain conditions the streptokinase-plasminogen-fibrinogen system can behave like plasma fibrinolysin in becoming demonstrable only after the fibrinogen has clotted, and that Williams (1951) suggested that the fibrinolytic activity of urine noted by Macfarlane & Pilling (1947) might be due to a kinase capable of activating plasminogen present as a contaminant in the substrate.

SUMMARY

1. Fibrinolytic plasma clots made in the presence of free calcium lyse less rapidly than those made in its absence. The effect is not due to activation by excess citrate nor, probably, to a direct inhibition by calcium ions.

2. Fibrin clots made from exercise plasma remain constant in amount for a variable time and then lyse completely within a comparatively short time.

3. In contrast to plasmin prepared by chloroform activation, samples of lysin from living subjects and from cadavers were not inhibited by normal plasma and did not digest fibrinogen.

4. The ratios of activities of given quantities of cadaver lysin and plasmin on fibrinogen and plasma clots were different.

5. Cadaver lysin and plasmin could not be differentiated with regard to optimum pH of activity and stability; both were found in the euglobulin fraction.

6. Fibrinolysin in plasma from living subjects and cadaver lysin may differ only quantitatively; neither is identical with plasmin. Attempts to demonstrate a kinase have been inconclusive.

I am grateful to Dr W. E. van Heyningen for purified streptokinase, to Mr H. Proom of the Wellcome Research Laboratories for refined anti-streptococcal serum, to the volunteers who supplied me with 'exercise plasma' and to members of the staff of the Radcliffe Infirmary who collected specimens of post-mortem blood. It is a pleasure to record the constant help and encouragement given to me by Dr R. G. Macfarlane. This work was financed by the Nuffield Foundation Haematological Research Fund.

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The Incorporation of Labelled Phosphate into the Lipids of a Brain Dispersion

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It is now known from isotopic investigations with labelled phosphate that the phospholipid phosphorus present in adult brain tissue is in a state of dynamic equilibrium with the inorganic phosphate in the cerebral cells. On the basis of results obtained by the intravenous injection of labelled phosphate, it was originally thought that this phosphorus exchange of the brain phospholipids was extremely slow. However, when a correction is made for the very slow rate at which phosphate ions present in the circulation pass into the brain (Dawson, 1952) the rate of phospholipid phosphorus exchange becomes appreciable and comparable with that of other tissues.

In spite of the obvious importance of this process in the nervous system, it has been little investigated. Fries, Schachner & Chaikoff (1942) demonstrated that rat-brain slices could incorporate labelled phosphate into their lipids which suggested that part at least of the radioactive brain phospholipids found after *in vivo* isotopic experiments was actually synthesized in cerebral tissue. A continuation of these studies (Schachner, Fries & Chaikoff, 1942) showed that the uptake was accelerated by hexoses and largely abolished by anaerobic conditions, suggesting that the incorporation was associated with the respiration of the tissue. However, on disruption of the cellular organization by 'homogenization' the rate of uptake was greatly reduced and was no longer changed on the addition of glucose to the incubation medium.

The advantage of using tissue dispersions for investigating the process of phospholipid phosphorus exchange is that factors of cellular permeability do not complicate the interpretation of the results. It has been found in the present investigation that freshly prepared and correctly fortified cell-free dispersions of guinea-pig brain readily incorporate labelled phosphate into their lipids, at a rate which is easily measured by conventional counting methods. A technique has been developed by which the lipid fraction can be easily extracted and freed from contaminating inorganic phosphate and acid-soluble phosphate esters of high specific activity which are formed during the incubation. The purification is therefore better than methods which depend upon dilution of the contaminating radioactivity with inorganic phosphate. Hahn & Tyrén (1946) have shown that these dilution methods are unsatisfactory for removing contaminating radioactivity from lipid extracts of liver.

The results of experiments with brain dispersions show beyond reasonable doubt that the replacement of lipid phosphorus is a process which is dependent upon an active oxidative phosphorylation in the tissue, which makes it extremely unlikely that the incorporation of radioactive phosphorus (^{32}P) is due to the reversibility of the enzymic processes by which phospholipids are hydrolysed into inorganic phosphate and other simpler components. These hydrolytic enzymes are appreciably active in brain tissue (Sloane-Stanley, 1952) and consequently no net increase in total phospholipid can be detected.

It was observed by Friedkin & Lehninger (1949*a*) that the incorporation of labelled phosphate into the lipids of a liver particulate preparation was accelerated when the preparation was actively respiring with malate as substrate so that the phosphate was rapidly esterified. In a recent note Kennedy (1952) has reported that the uptake of phosphate into the lipids of isolated liver mitochondria is inhibited by the presence of 2:4-dinitrophenol.

A preliminary communication about this work has already been presented to the Biochemical Society (Dawson, 1953).

METHODS

Preparation of dispersions and incubation. A young adult male guinea pig was stunned, decapitated, and its brain rapidly dissected out and weighed. A dispersion of the whole brain was prepared by grinding in an ice-cold mortar according to the description of Banga, Ochoa & Peters (1939). After thoroughly grinding the brain into a uniform paste, a cold solution of 0·08 M-KCl and 0·12 M-NaF was very gradually worked into the paste until the dispersion was of the requisite dilution. It was then filtered through muslin or glass wool and stored until required at 0°, its ability to respire and to incorporate phosphate into its lipids being reasonably stable under these conditions. Histological examination of these dispersions by Mr E. H. Leach showed them to be virtually cell-free, although they

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contained intact nuclei and mitochondria, together with fibrils and innumerable small particles. After incubation for 1 hr. in the presence of a substrate the mitochondria became less numerous and the number staining with Janus green decreased. Brain dispersions prepared in a Waring blendor showed a low capacity for incorporating phosphate into their lipids.

Samples of the dispersion were pipetted into Warburg vessels containing the incubation medium and labelled phosphate (approx. 6 μc) and 0·2 ml. 10% KOH in the centre cup. The vessels were immediately transferred to a Warburg bath and shaken at 37°. Respiration readings were commenced at 6 min. by which time the contents had attained temperature equilibrium with the bath. In experiments in which the carrier-free phosphate was tipped from the side arm the results were generally inconsistent. This is probably due to variable adsorption of the labelled phosphate on the glass (Harrison & Raymond, 1951; Lamerton & Hariss, 1951). At the end of the incubation 5–10 ml. of 20% (w/v) trichloroacetic acid were added to the contents of each flask. This destroys some of the plasmalogens which are unstable to acid even at room temperature (Feulgen & Bersin, 1939; Hack, 1947), but it is an important factor in ensuring that the final lipid extract is free from acid-soluble phosphate esters of high specific activity.

Filtration, extraction and washing of the lipids. The filtration of the precipitated lipids and proteins, and the subsequent solvent extraction of the lipids, were both carried out in the same Soxhlet extraction vessel. A series of these vessels were mounted as illustrated in Fig. 1. Each extractor of about 15 ml. capacity was tightly packed with glass wool to a depth of 2·5 cm., followed by a loose layer of glass wool. The mixture was transferred to the extractor with the aid of a funnel, when the trichloroacetic acid extract percolated through the glass wool, siphoning over into the bulb, where it collected as a clear filtrate. A sample of this filtrate was removed when required for examination of the acid-soluble phosphorus. Filtration was rapid provided that no more than 0·14 g. brain tissue which had been precipitated with trichloroacetic acid was filtered through 1 sq.cm. of the filter. The precipitate was then washed with about 60 ml. of 5% (w/v) trichloroacetic acid solution followed by 60 ml. of water; the final washings had a pH of 3–4. The end of a bent glass rod covered with soft rubber tubing was then inserted into the vapour orifice of the extractor, and air sucked through the precipitate until it was free from surplus supernatant. Subsequently the precipitate was extracted for 5 hr. with a mixture of equal volumes of $CHCl_3$ and methanol. Siphoning of the solvent was normal provided the bore of the siphon tube was not too narrow. At first troublesome and violent bumping of the solvent was experienced, and this was not eliminated by the usual remedies. It was then found that if the extraction flasks were washed after each run by refluxing $CHCl_3$: methanol in them a layer of insoluble matter gradually collected on the glass, which completely cured any tendency to bump. After extraction the solution of lipids was cooled, and poured into a separating funnel containing 60 ml. of N-HCl. The flask was washed with two 5 ml. portions of $CHCl_3$ which were added to the separating funnel. This was shaken mechanically for 5 min. and the lower $CHCl_3$ layer which separated rapidly was filtered through a Whatman no. 2 paper. The aqueous phase contained the contaminating acid-soluble phosphorus and experiments showed that further washing of the $CHCl_3$ layer with N-HCl did not remove any more radioactivity. Suspended in the aqueous phase was a curdy, white precipitate which was initially soluble in a $CHCl_3$: methanol mixture, but on standing in HCl became insoluble. Initially this precipitate contained a little phosphorus which had a specific activity equal to that of the lipid fraction, but on standing and then washing with ether the phosphorus content became negligible (<0·1%). When the precipitate was hydrolysed in 6 N-HCl and a two-dimensional chromatogram of the hydrolysate prepared, about fourteen amino acids could be detected by ninhydrin spraying, including large amounts of alanine and leucine isomers. It is therefore highly probable that the precipitate contains proteolipids whose presence in brain has recently been described by Folch & Lees (1951).

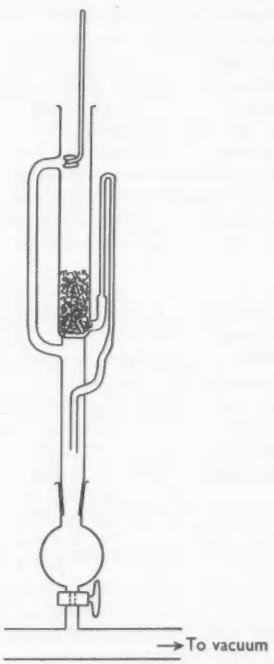

Fig. 1. Filtration and extraction unit.

After the $CHCl_3$ layer had been evaporated to dryness the lipids were oxidized and assayed for radioactivity by methods previously described (Dawson & Richter, 1950). Phosphorus estimations on duplicate samples were carried out by the method of Fiske & Subbarow (1929), the colour being read in a Beckman spectrophotometer at 660 mμ.

Materials

Glycylglycine. The commercial product (10 g.; British Drug Houses Ltd.) was dissolved in 48 ml. water, filtered under pressure, and 240 ml. ethanol slowly added to the filtrate. The precipitate was filtered, washed with ethanol and dried at room temperature. This reduced the inorganic phosphorus content to below 0·01%. The peptide was then dissolved in water and titrated to pH 7·2 with KOH.

Sodium pyruvate. This was prepared by a method based on the observations of Peters (1936) and Lipschitz, Potter & Elvehjem (1938). Pyruvic acid doubly distilled *in vacuo* was diluted with an equal volume of water and neutralized to pH 5 with NaOH solution, keeping the temperature below 30°. The salt was then crystallized by adding acetone until precipitation just commenced, followed by cooling to 0°. The sodium pyruvate was recrystallized at frequent intervals by the method of Robertson (1942).

Cytochrome *c* was prepared by the method of Keilin & Hartree (1937), but in later experiments a commercial preparation (Light & Co., Slough) was used.

The adenosine triphosphate (ATP) and diphosphopyridine nucleotide (DPN) used were samples prepared in this laboratory by Dr L. A. Stocken.

Radioactive phosphate. It was found that even traces of microflora growing in the stock carrier-free labelled phosphate solution greatly increased the zero-time 'incorporation' values. It could be stored satisfactorily for several weeks by diluting to a convenient volume with sterile water and storing in a closed container at 0°. Before pipetting the labelled phosphate solution into the Warburg vessels the pipette was rinsed six times with the solution to minimize adsorption effects.

Expression of results. The incorporations observed have been expressed in terms of the specific activities of the isolated lipid fraction, as this eliminates any error in the quantitative isolation of the lipids. In no experiment did the actual concentration of the lipid phosphorus alter sufficiently to change appreciably the specific activity of the fraction. It was calculated that the specific activity of the lipid phosphorus in an extract prepared from a brain dispersion respiring for 1 hr. in a complete medium could be measured with an accuracy of $\pm 4\%$.

RESULTS

The efficiency of the preparation of lipid

The method used in the present investigation to prepare lipid phosphorus for radioactive assay was designed to enable the lipid to be easily obtained from brain tissue in good yield and free from acid-soluble phosphorus. Experiments showed that only a further 1% more lipid phosphorus was obtained on prolonging the solvent extraction to 15 hr. The precipitation with trichloroacetic acid resulted in the loss of about 5–15% of the total phospholipid phosphorus present in the brain tissue. This loss was caused by the decomposition of a phosphorus-containing lipid which is estimated as sphingomyelin in the conventional method of estimation, and also by the release of some acid-soluble phosphorus from plasmologens. Measurements showed that less than 5% of the lipid phosphorus present in an extract was lost during the purification procedure.

Zero-time 'incorporation'. The extreme difficulty of completely freeing lipid extracts from contaminating acid-soluble phosphorus has been noted by Fries *et al.* (1942) and McKibbin & Taylor (1949). With the present method of purification about 99% of the radioactivity was removed from a lipid extract which had been deliberately contaminated with carrier-free labelled inorganic phosphate. In further experiments a solution of labelled acid-soluble phosphorus compounds was prepared from a brain dispersion which had been incubated with labelled phosphate. This solution was added to another dispersion which was then precipitated with trichloroacetic acid. A lipid extract of this precipitate after washing with hydrochloric acid possessed negligible radioactivity, showing that the labelled phosphorus compounds had been adequately separated from the lipids.

The specific activity of the lipid phosphorus isolated from a dispersion which had been immediately treated with trichloroacetic acid after adding labelled phosphate amounted to less than a few per cent of that isolated after incubation for 1 hr. in a complete medium. This residual, or zero-time, radioactivity appeared to be tenaciously 'bound' to the lipids and no easy method of removing it was discovered. It did not appear to be bound by enzymic action as it occurred with boiled dispersions and was increased somewhat by adding inorganic phosphate (0·005 M) to the medium. When labelled phosphate was added to a dispersion immediately after incubation for 1 hr. the zero-time 'incorporation' was appreciably less than that of the fresh dispersion, even after correcting for the differing concentrations of inorganic phosphate in the media. For this reason the values obtained in the present investigation have not been corrected for zero-time 'incorporation', as there is no evidence that such a correction would be valid.

Factors which affect the enzymic incorporation of labelled phosphate into the lipids

Initial experiments using dialysed dispersions showed a comparatively low rate of incorporation of labelled phosphate into the lipid fraction, and studies were therefore confined to the undialysed dispersions at various dilutions. It was found that the incorporation of phosphate occurred at a maximum rate during the first hour of an incubation at 37° the subsequent uptake being relatively slow (Fig. 2). The associated respiration also declined but it fell off much less rapidly than the incorporation. It is surprising that there is no apparent time lag at the beginning of the incorporation during which labelled precursors of the phospholipids are synthesized, and this must mean that such precursors attain a maximal specific activity very rapidly. In this connexion recent experiments have shown that labelled phosphate added to a kidney-cortex mitochondrial preparation reaches radioactive equilibrium with external ATP within a few minutes (Bartley & Davis, 1952). A similar rapid rate of exchange of the labile groups of ATP is found when

a liver dispersion is incubated with labelled phosphate (Ennor, 1952).

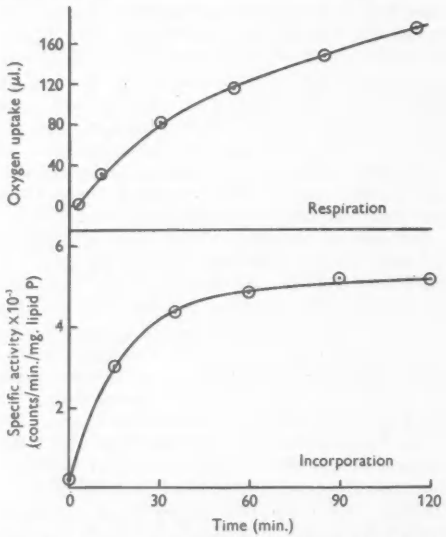

Fig. 2. The incorporation of labelled phosphate into the lipids of a guinea-pig brain dispersion. Medium: glycylglycine buffer, pH 7·2, 0·1 M; adenylate, 0·001 M; Mg^{2+}, 0·017 M; pyruvate, 0·03 M; fumarate, 0·0033 M; F^-, 0·017 M; cytochrome *c*, $3·2 \times 10^{-5}$ M; ^{32}P, 10^6 counts/min. 240 mg. fresh brain in 3 ml.; 37°.

The composition of the medium used in the present investigation was based on the experiments of Case & McIlwain (1951) who investigated the conditions necessary for optimal phosphorylation and respiration in brain dispersions. However, glucose, ATP and some of the phosphate were usually omitted, because in general these were found to reduce the incorporation of labelled phosphate into the lipids. The incubations were carried out in the presence of fluoride which markedly increased the ^{32}P uptake. The rate of incorporation did not change appreciably when veronal or bicarbonate:carbon dioxide buffers replaced the glycylglycine buffer usually employed.

Additions to the incubation medium which were found necessary to obtain a maximum incorporation of labelled phosphate into the lipids are shown in Table 1 which presents the results of two typical experiments. The effects shown were all confirmed on two or three independent brain dispersions. For maximum incorporation the system requires the presence of an oxidizable substrate, magnesium ions, cytochrome *c*, adenylic acid and oxygen. It seems likely that these factors are necessary to ensure a high rate of oxidative phosphorylation and consequent esterification of the ^{32}P present in the incubation medium. It was shown by Friedkin & Lehninger (1949*b*) that both magnesium ions and cytochrome *c* are required in the system which esterifies inorganic phosphate during the passage of electrons from reduced diphosphopyridine nucleotide to oxygen. In the present experiments it was found that when magnesium ions were left out of the medium there was usually a slight stimulation of the respiration which was not observed when a phosphate buffer was also present in the medium. The presence of adenylic acid is probably necessary to ensure a supply of suitable phosphate acceptor (adenosine diphosphate, ADP) through the myokinase system (Ochoa, 1944; Hunter, 1949). On the addition of a limited amount of ATP to a dilute dispersion (40 mg. brain/ml.) a slight stimulation of the respiration was observed and this was usually

Table 1. *The incorporation of labelled phosphate into the lipids of a guinea-pig brain dispersion*

(Medium: glycylglycine buffer, pH 7·2, 0·1 M; adenylate, 0·001 M; Mg^{2+}, 0·017 M; pyruvate, 0·03 M; fumarate, 0·0033 M; F^-, 0·019 M; cytochrome *c*, $3·2 \times 10^{-5}$ M; ^{32}P, 10^6 counts/min. 120 mg. fresh brain in 3 ml. total vol., incubated at 37° for 1 hr. in air.)

		Respiration		Specific activity lipid P	Specific activity
Experiment	Medium	(μl.)	% of that with complete medium	(counts/min./ mg. P)	(% of that with complete medium)
1	Complete	77·5	100	12 600	100
	No Mg	87	112	5 680	45
	No adenylate	79	102	10 650	84
	No cytochrome *c*	86	111	7 580	60
	No pyruvate or fumarate	51·5	66	2 760	22
	Unfortified	67	86	2 260	18
	Complete + ATP, $2·5 \times 10^{-4}$ M	80	103	13 700	109
2	Complete	77	100	12 800	100
	No fumarate	66	86	4 400	34
	No cytochrome *c*	75	97	9 900	77
	No incubation	—	—	79	0·6
	Complete + glucose, 0·031 M	79	102	3 820	30
	Complete + glutamate, 0·011 M	92	120	14 800	116

Table 2. *Effect of adenosine triphosphate on the incorporation of labelled phosphate into the lipids of a guinea-pig brain dispersion*

(Medium: glycylglycine buffer, pH 7·2, 0·05 M; adenylate, 0·001 M; Mg^{2+}, 0·017 M; pyruvate, 0·03 M; fumarate, 0·0033 M; cytochrome *c*, $2·2 \times 10^{-5}$ M; phosphate buffer, pH 7·2, 0·003 M; F^-, 0·022 M; ^{32}P, 10^6 counts/min. 240 mg. brain in 3 ml. total vol., incubated at 37° in air.)

ATP added* ($M \times 10^{-4}$)	Time of incubation (hr.)	Final concn. inorganic P ($M \times 10^{-3}$)	Respiration (μl.)	Specific activity lipid P (counts/min./mg. P)
0	0	3·75	—	256
0	1	2·49	180	6850
2·5	1	2·61	205	5720
5·1	1	2·59	207	5450
7·7	1	2·93	218	4320
12·2	1	3·00	196	4010

* ATP was a pure sample with a low inorganic pyrophosphate content; labile phosphate was determined by 10 min. hydrolysis in N-HCl.

associated with an increase in the rate of incorporation of ^{32}P into the lipids. At higher concentrations of ATP or on adding ATP to less dilute dispersions (80 mg./ml.) there was a definite inhibition of ^{32}P uptake (Table 2). It would seem, therefore, that because of the rapid post-mortem depletion of ATP (Maleci, 1950) it is necessary to add a limited amount to dilute dispersions to obtain a satisfactory concentration of ADP for phosphate acceptance. Above this concentration it inhibits the uptake into the lipids either by a direct isotopic dilution effect or by breakdown into inorganic phosphate which lowers the specific activity of the labelled phosphate from which the labelled phospholipid precursors are synthesized.

The addition of a coenzyme A concentrate (20 units) to a respiring dispersion (80 mg. brain/ml.) produced no appreciable change in the incorporation of ^{32}P into the lipids. Two DPN samples assaying at 40 and 85 % pure both produced a slight inhibition of the incorporation when added at a concentration of 0·001 M.

Influence of substrates. The necessity of added oxidizable substrate for the incorporation of ^{32}P into the lipids is clearly seen in Table 1. It is surprising that the incorporation is inhibited far more than the respiration by the absence of added substrate. This could be due to utilization of residual substrates in the brain homogenates whose oxidation is not so favourably coupled with the esterification of ^{32}P as the oxidation of pyruvate plus fumarate. Glucose at low concentrations had a pronounced inhibitory action on the incorporation so that when present as the sole added substrate the incorporation was less than 10 % of that with pyruvate plus fumarate as substrate (Table 3). This inhibitory effect of glucose was also observed in the presence of pyruvate plus fumarate (Table 1), and it is therefore likely that, as hexokinase is present in the dispersion, the glucose is competing with phospholipid precursors for the labelled phosphate bonds of ATP. A similar but smaller inhibition of the incorporation was observed on adding 2×10^{-2} M-creatine to the incubation medium, and it is probable that here the creatine is competing with phospholipid precursors for the labelled phosphate bonds of ATP through the Lohmann reaction.

Table 3. *Effect of different substrates on the incorporation of labelled phosphate into the lipids of a guinea-pig brain dispersion*

(Medium: glycylglycine buffer, pH 7·2, 0·1 M; phosphate buffer, pH 7·2, 0·001 M; adenylate, 0·001 M; Mg^{2+}, 0·017 M; F^-, 0·019 M; cytochrome *c*, $3·2 \times 10^{-5}$ M; $^{32}P = 10^6$ counts/min. 120 mg. fresh brain in 3 ml. total vol., incubated at 37° for 1 hr. in air.)

Substrate	Respiration (μl.)	Specific activity lipid P (counts/min./mg. P)
Pyruvate, 0·03 M + fumarate, 0·0033 M	86	6210
Fumarate, 0·02 M	76	5290
α-Ketoglutarate, 0·02 M	103	3880
Glutamate, 0·02 M	97	5700
Malate, 0·02 M	96	7680
Succinate, 0·02 M	240	2950
Glucose, 0·02 M	50	540

The use of glutamate as an oxidizable substrate resulted in a rate of incorporation similar to that found with pyruvic plus fumarate as substrate (Table 3) which agrees with the satisfactory utilization of glutamate as a substrate for oxidative phosphorylation by cerebral mitochondria (Abood & Gerard, 1952; Brody & Bain, 1952). On adding glutamate to a dispersion respiring with pyruvate plus fumarate as substrate there was a slight stimulation of both the respiration and the incorporation. The possibility that this increased incorporation was due to an inhibition of phosphatase action by glutamic acid (Bodansky, 1948) was considered, but experiments showed that the addition of histidine had no stimulatory action on the incorporation. The utilization of succinate as oxidizable substrate

resulted in a high rate of respiration, but the incorporation was only about half that obtained with pyruvate plus fumarate as substrate (Table 3). This is in keeping with the results of Ochoa (1941) and Brody & Bain (1952), who found that cerebral particulate preparations had a high rate of respiration and a low P/O ratio when succinate was the substrate. The succinic-oxidase system is known to be very stable on incubation at 37°, but at the same time the oxygen uptake becomes uncoupled from phosphorylation.

Anaerobic conditions. In anaerobic experiments the Warburg vessels were flushed with nitrogen and shaken for 5 min. with yellow phosphorus in the centre cup. The labelled phosphate was then tipped into the main compartment from the side arm. The results of anaerobic experiments in which the dispersions used were prepared with and without fluoride are presented in Table 4. Anaerobiosis

Table 4. *Anaerobic glycolysis and the incorporation of labelled phosphate into the lipids of a guinea-pig brain dispersion*

(Medium: glycylglycine buffer, pH 7·2, 0·1 M; adenylate, 0·001 M; Mg^{2+}, 0·017 M; fumarate, 0·0033 M; cytochrome *c*, $3{\cdot}2 \times 10^{-5}$ M; ^{32}P, 10^6 counts/min. 240 mg. fresh brain in 3 ml. total vol., incubated at 37° for 1 hr.)

	Specific activity lipid P (counts/min./mg. P)	
Conditions	No fluoride	+0·022 M Fluoride
Glucose (0·0154 M), aerobic	1060	1830
Glucose (0·0154 M), anaerobic	470	520
No glucose, anaerobic	200	450

resulted in a considerable reduction in the rate of incorporation of labelled phosphate into the lipids but the lowered rate of incorporation was increased somewhat by the addition of glucose. The stimulation produced by glucose was greatest in the absence of fluoride. This may be due to the higher rate of glycolysis in the dispersion producing a lower residual glucose. Although the quantitative interpretation of the results is complicated by the inhibitory action of glucose on the incorporation, there seems little doubt that anaerobic glycolysis can to a limited extent support the uptake of ^{32}P into the lipids. The use of yellow phosphorus resulted in a high degree of anaerobiosis, but it was shown that the phosphorus pentoxide fumes often resulted in a transfer of phosphorus into the medium. Presumably the metaphosphate formed would be quickly converted into orthophosphate but it was calculated that the amount transferred in the present experiments would not significantly affect the results obtained.

Addition of fluoride. When a dispersion was incubated with pyruvate plus fumarate as substrate, the addition of fluoride resulted in a marked increase in the incorporation of labelled phosphate into the lipids and a decrease in the respiration. The curve relating fluoride concentration and incorporation (Fig. 3) reaches a maximum at 0·02 M, and its general shape is very similar to that obtained by Case & McIlwain (1951) when they determined the relationship of fluoride concentration and phosphorylation in brain dispersions. It is therefore likely that fluoride inhibits the active adenosine triphosphatase present in brain tissue and thus prevents the breakdown of labelled ATP enabling it to be used for phosphorylating precursors of the phospholipids.

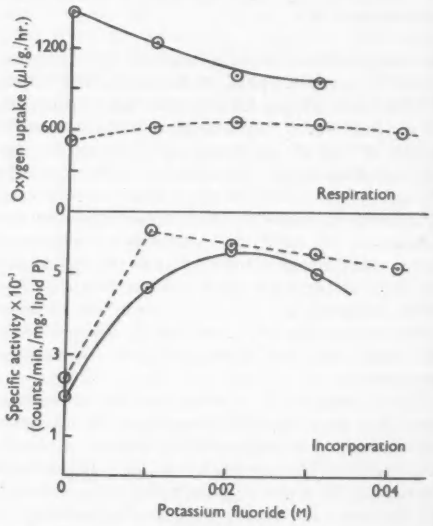

Fig. 3. The effect of fluoride on the incorporation of labelled phosphate into the lipids of a guinea-pig brain dispersion. Medium: glycylglycine buffer, pH 7·2, 0·1 M; with and without phosphate buffer, pH 7·2, 0·003 M; with and without adenylate, 0·001 M; Mg^{2+}, 0·017 M; pyruvate 0·03 M; fumarate 0·0033 M; ^{32}P, 10^6 counts/min. 240 mg. fresh brain in 3 ml., 1 hr., 37°. ⊙—⊙, Adenylate and phosphate buffer omitted; ⊙—⊙, adenylate and phosphate buffer added.

When adenylic acid and phosphate were omitted from the incubation medium it was observed that although there was markedly increased incorporation on adding fluoride, the oxygen uptake was not inhibited, but actually slightly increased (Fig. 3). Further experiments indicated that the decreased respiration on adding fluoride was only observed if adenylic acid was present in the medium and that the inhibition was independent of the presence of a phosphate buffer.

Table 5. *Effect of inhibitors on the incorporation of labelled phosphate into the lipids of a guinea-pig brain dispersion*

(Medium: glycylglycine buffer, pH 7·2, 0·1 M; adenylate, 0·001 M; Mg^{2+}, 0·017 M; phosphate buffer, pH 7·2, 0·001 M; pyruvate, 0·03 M; fumarate, 0·0033 M; F^-, 0·022 M; cytochrome *c*, $2·1 \times 10^{-5}$ M; ^{32}P, 10^6 counts/min. 240 mg. fresh brain in 3 ml. total vol., incubated at 37° for 1 hr. in air.)

Inhibitor	Concentration (M)	Increase (+) or decrease (−) in respiration (%)	Incorporation of ^{32}P into lipids. Inhibition (%)
Arsenate	$4·4 \times 10^{-3}$	+5	32
	$8·8 \times 10^{-3}$	+6	42
Azide	10^{-3}	+4	78
Calcium chloride	1×10^{-3}	−16	77
	$3·5 \times 10^{-3}$†	−45	89
2:4-Dinitrophenol	10^{-4}	+81	47
Gramicidin	5×10^{-6}‡	+28	66
Iodoacetate	3×10^{-3}	−16	21
Malonate	$4·3 \times 10^{-3}$	−3	16
	$4·3 \times 10^{-2}$	−26	45
Methylene blue	$2·5 \times 10^{-4}$	+2	77
	10^{-3}	−24	81
2-Octanol	$3·6 \times 10^{-2}$ added	−86	92
Phlorrhizin	$4·5 \times 10^{-3}$	−57	72
Potassium cyanide*	10^{-3}	−48	76
Potassium sulphate	$3·3 \times 10^{-2}$	+20	23

Respiration of control dispersions = 126–148 μl.
Specific activity of lipids of control dispersions = 5710–7225 counts/min./mg. P.

* KCN/KOH in centre Warburg cup.
† Phosphate buffer 0·003 M.
‡ 0·015 ml. 10^{-3} M in 66% ethanol.

When adenylic acid is present, the myokinase in the dispersion will ensure that the respiration will not be inhibited by the lack of phosphate acceptor (ADP), but on the addition of fluoride the inhibition of myokinase produced (Barkulis & Lehninger, 1951) will limit ADP formation and so inhibit respiration. In the absence of adenylic acid little or no formation of ADP by myokinase action will occur, and the respiration will be limited by the lack of a phosphate acceptor. Under these conditions the addition of small quantities of fluoride is not likely to have an appreciable inhibitory effect on the respiration. The fact that there is a slight stimulation of the oxygen uptake could possibly mean that the removal of the limited supplies of ADP by myokinase is inhibited more than its formation by the action of adenosine triphosphatase (Ochoa, 1944; Hunter, 1949; Barkulis & Lehninger, 1951).

Action of inhibitors. The effects of various inhibitors on the respiration and incorporation of labelled phosphate into the lipids of brain dispersions are shown in Table 5. These experiments were all conducted in the presence of fluoride and with pyruvate plus fumarate as substrate. Inhibitors of respiration, namely cyanide, malonate, iodoacetate, octanol and phlorrhizin all caused a reduction of the incorporation of ^{32}P which was always greater than the corresponding inhibition of the respiration. Agents which can cause an uncoupling of oxidative phosphorylation, e.g. arsenate, azide, 2:4-dinitrophenol, gramicidin, methylene blue and sulphate ions, all produced at a suitable concentration a stimulation of the respiration, and a reduction in the incorporation. In these experiments with inhibitors measurements were made of the inorganic phosphate plus labile phosphate present in the final incubation mixture, and in no instance did the concentration differ appreciably from that of a corresponding control.

The effect obtained on adding calcium ions to a dispersion is also shown in Table 5, and it is seen that even at a normal serum concentration of ionizable calcium (10^{-3} M) there was a profound inhibition of the incorporation with a slight reduction in the rate of oxygen uptake. Keltch & Clowes (1951) have shown that a low concentration of calcium inhibits the aerobic phosphorylation of glucose in particulate brain preparations, with little or no effect on the respiration.

Tonicity of incubation medium. When potassium or sodium chloride was added to an incubation medium, which was already isotonic, there was a marked inhibition of the incorporation of labelled phosphate into the lipids (Fig. 4). The effect on the respiration was far less marked and an inhibition only became apparent when the tonicity had increased by about 50%. Increasing the tonicity by the addition of sucrose also produced a decreased incorporation, but the inhibition was much less than that found with the electrolytes. Lehninger

(1949) has observed that the coupled phosphorylation of liver mitochondria is dependent upon the tonicity of the incubation medium, and Brody & Bain (1952) have noticed that cerebral mitochondria prepared in hypertonic sucrose (0·88 M) had very low P/O ratios. On the other hand, Slater & Cleland (1953) have recently reported that the P/O ratio of the α-ketoglutaric-oxidase system in heart-muscle preparations is only slightly reduced when the tonicity is increased by the addition of saline.

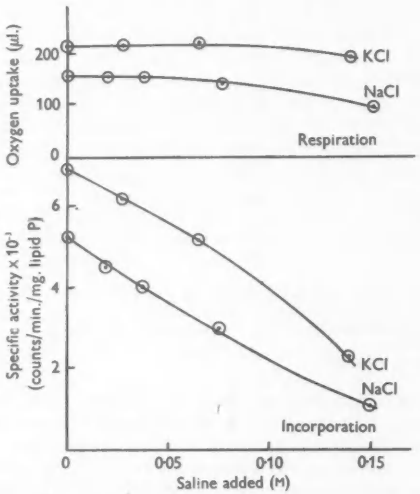

Fig. 4. Effect of hypertonic saline on the incorporation of labelled phosphate into the lipids of guinea-pig brain dispersion. Medium: glycylglycine buffer, pH 7·2, 0·05 M; phosphate buffer, pH 7·2, 0·003 M; adenylate, 0·001 M; Mg^{2+}, 0·017 M; pyruvate, 0·03 M; fumarate, 0·0033 M; F^-, 0·022 M; cytochrome *c*, $2·1 \times 10^{-5}$ M. ^{32}P 10^6 counts/min. 240 mg. fresh brain in 3 ml., 1 hr., 37°.

Optimum pH. The optimum pH for the incorporation was measured using low molarity phosphate buffers (Na_2HPO_4/KH_2PO_4) in which the phosphate concentration was kept constant. Each medium was approximately adjusted to the required pH before the introduction of the buffer. It is seen from Fig. 5 that the pH optimum is near 6·5 and different from that of the respiration.

Phosphate concentration. Addition of inorganic phosphate to the incubation media reduced the incorporation of ^{32}P into the lipids. At the same time there was a stimulation of the oxygen uptake, and this probably accounts for the deviation of the curve plotting incorporation against phosphate concentration (Fig. 6) from that predicted by only considering the changing specific activity of the labelled phosphate precursor. It was consistently observed that the zero-time 'incorporation' of

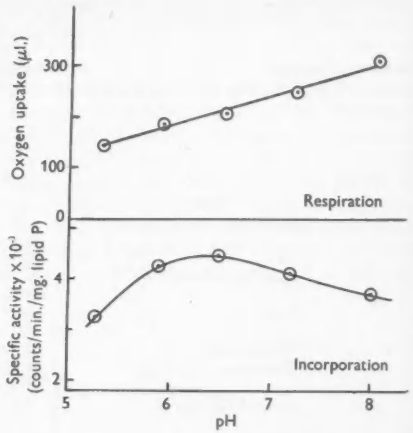

Fig. 5. Effect of pH on the incorporation of labelled phosphate into the lipids of a guinea-pig brain dispersion. Medium: phosphate buffer, 0·003 M; adenylate, 0·001 M; Mg^{2+}, 0·017 M; pyruvate, 0·03 M; fumarate, 0·0033 M; F^-, 0·022 M; ^{32}P, 10^6 counts/min. 240 mg. fresh brain in 3 ml., 1 hr., 37°.

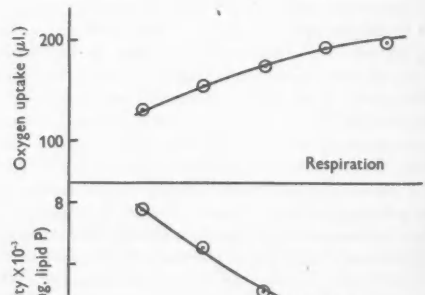

Fig. 6. Effect of initial phosphate concentration on the incorporation of labelled phosphate into the lipids of a guinea-pig brain dispersion. Medium: glycylglycine buffer, pH 7·2, 0·1 M; adenylate, 0·001 M; Mg^{2+}, 0·017 M; malate, 0·011 M; cytochrome *c*, $2·1 \times 10^{-5}$ M; F^-, 0·022 M; ^{32}P, 10^6 counts/min. 240 mg. fresh brain, 1 hr., 37°.

labelled phosphate became higher when the inorganic phosphate concentration in the medium was increased. During the present investigation the concentration of phosphate which was added to the incubation medium in some experiments never exceeded the *in vivo* concentration of inorganic phosphate in the brain.

Addition of possible phospholipid precursors. Experiments in which various possible precursors of the phospholipids were added to the incubation medium are reported in Table 6. None of these additions produced an appreciable increase in the rate of ^{32}P incorporation into the lipid fraction, and it is therefore concluded that if any of these substances are required for the synthesis of labelled phospholipids they are present in sufficient concentration in the dispersion or they are unable to penetrate to the active centre of synthesis. The effect of oleate at a low concentration (2×10^{-3} M) is interesting, as it inhibits the respiration and even more markedly the incorporation. Peters & Wakelin (1938) have reported a similar inhibition of the respiration of a pigeon-brain brei on adding sodium oleate adjusted to pH 7·4. Moreover, the inhibitory action of fatty acids on the fatty-acid oxidase system is thought to be due to their toxic effect on some phase of oxidative phosphorylation (Lehninger, 1951). It is possible, therefore, that the soluble soap has a powerful destructive effect on the biochemical organization of the particulate matter in cell-free brain preparations. It is surprising, however, that under the present conditions of incubation, stearate had no similar action on the respiration or incorporation.

Table 6. *Effect of adding possible precursors on the incorporation of labelled phosphate into the lipids of a guinea-pig brain dispersion*

(Medium: glycylglycine buffer, pH 7·2, 0·05 M; adenylate, 0·001 M; Mg^{2+}, 0·017 M; malate, 0·02 M; F^-, 0·022 M; cytochrome *c*, $2·1 \times 10^{-5}$ M; phosphate buffer, pH 7·2, 0·004 M; ^{32}P, 10^6 counts/min. 240 g. fresh brain in 3 ml. total vol., incubated at 37° for 1 hr. in air.)

Substance	Concentration (10^{-3} M)	Respiration (% of control)	Specific activity of lipid P (% of control)
Control	—	100	100
Choline	8	94	102
Betaine	8	95	100
Ethanolamine	8	96	108
L-Serine	8	99	106
Inositol	8	97	102
Glycerol	8	100	102
Stearate	3	119	110
Oleate	2	59	17

Respiraton of controls = 199–264 μl.
Specific activity of lipid P of controls = 3355–3610 counts/min./mg. P.

On the addition of either α- or β-glycerophosphate at a concentration of 0·0025 M there was no appreciable change in the rate of incorporation of ^{32}P from a labelled 0·005 M-phosphate buffer. Although these phosphate esters both caused a slight stimulation of the respiration (see Peters & Sinclair, 1933) there is no evidence that they are penetrating to the active centres of phospholipid synthesis. From the results of experiments on the *in vivo* turnover of phospholipids in rat liver Popják & Muir (1950) have concluded that glycerophosphates are precursors of liver lecithin and kephalin, but they were unable to obtain confirmation of this from *in vitro* experiments. In a recent note Kennedy (1952) has reported that unlabelled glycerophosphate inhibited the incorporation of ^{32}P into the phospholipids of isolated liver mitochondria.

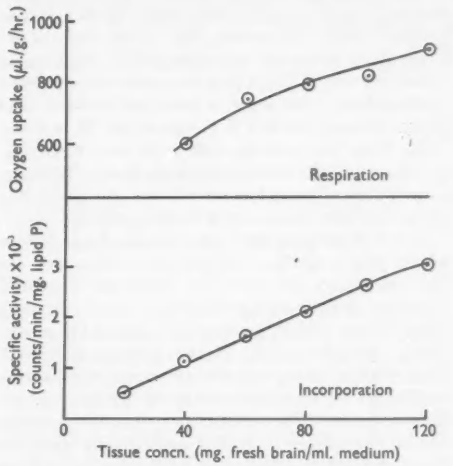

Fig. 7. Effect of tissue concentration on the incorporation of labelled phosphate into the lipids of a guinea-pig brain dispersion. Medium: glycylglycine buffer, pH 7·2, 0·1 M; ATP, 0·00025 M; Mg^{2+}, 0·017 M; pyruvate, 0·015 M; fumarate, 0·0033 M; F^-, 0·011 M; ^{32}P, 10^6 counts/min./g. fresh brain tissue. Incubated 1 hr. at 37°.

Tissue concentration. It is seen from Fig. 7 that both the respiration and the incorporation of ^{32}P into the lipids per unit weight of tissue increased with increasing tissue concentration. No stimulation of the uptake was observed on adding to the incubation medium an aqueous extract prepared from a boiled brain dispersion.

DISCUSSION

The present investigation has indicated that the incorporation of labelled phosphate into the brain lipids is associated with oxidative phosphorylation.

The technique of separation used ensures that the lipid fraction is adequately separated from contaminating acid-soluble phosphorus of high specific activity. There seems little doubt therefore that the active lipids isolated represent a genuine conversion of labelled phosphate into substances soluble in organic solvents. It is highly likely, but not proven, that this represents a synthesis of complete phospholipid molecules or their immediate precursors (e.g. phosphatidic acids). No net synthesis of lipid phosphorus could be detected, presumably because of the presence in the dispersion of catabolic enzymes which attack the phospholipids (Sloane-Stanley, 1952). However, the initial rate of incorporation observed in a dispersion respiring in a medium containing a physiological concentration of phosphate represents a minimal formation of 44 mg. phospholipids/100 g. tissue/hr. It is interesting that the corresponding *in vivo* figure for mouse brain calculated using a number of assumptions and approximations was 65 mg. phospholipids/100 g. tissue/hr. (Dawson & Richter, 1950).

It is a significant fact that many of the diverse agents which produce degenerative lesions of the nervous system are of a type which are known to interfere with energy-giving reactions in nervous tissue. They include, for example, anoxia (Morrison, 1946), cyanide, azide, carbon monoxide (Hurst, 1940, 1942), malononitrile (Hicks, 1950) and a deficiency of vitamins of the B group (Gildea, Kattwinkel & Castle, 1930). It is therefore possible that the interference with energy-giving reactions found in these conditions is inhibiting the normal dynamic replacement of such structural elements as phospholipids and proteins (see Peterson & Greenberg, 1952), and this may in turn initiate the production of degenerative lesions. The generation of such lesions in the central nervous system by feeding unnatural fats may also depend on such a blocking of the normal replacement of lipid structural elements (Schaltenbrand & Schorn, 1948).

SUMMARY

1. A simple technique has been devised for preparing a lipid extract of brain tissue containing a minimum of contaminating acid-soluble phosphorus.

2. The conditions have been determined for the maximum incorporation of labelled phosphate into the lipids of a guinea-pig brain dispersion.

3. For optimum incorporation the process requires oxidizable substrate, oxygen, phosphate acceptor, magnesium ions and cytochrome *c*, and is greatly accelerated by the presence of fluoride ions.

4. Hypertonic media and low concentrations of calcium ions and glucose strongly inhibit the incorporation.

5. The use of inhibitors has shown that those which are known to uncouple oxidative phosphorylation also uncouple the respiration of a dispersion from the incorporation of labelled phosphate into its lipids.

6. It is concluded that the initial step in the incorporation is an esterification of the ^{32}P into high-energy phosphate compounds, which then phosphorylate phospholipid precursors.

The author wishes to thank Prof. Sir Rudolph A. Peters, F.R.S., for his continual advice and interest in this investigation. He is greatly indebted to Mr E. H. Leach for his histological investigation of suspensions and also to Mr V. Butlin and Mr R. Lincoln for valuable technical assistance.

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The Amino Acid Composition of Salmine

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During recent years several authors have investigated the amino acid composition of salmine, employing a variety of classical and modern methods of analysis. Reference may be made to Tristram (1947), Hamer & Woodhouse (1949), Block, Bolling, Gershon & Sober (1949), Velick & Udenfriend (1951) and Mills (1952). The analyses of these authors clearly show that salmine contains only the seven amino acids: glycine, valine, alanine, isoleucine, proline, serine and arginine; Velick & Udenfriend (1951) identified a small amount of glutamic acid in their salmine hydrolysates but regarded it as an impurity. A careful examination of all these analytical data shows that, whereas there is good agreement for the glycine and arginine contents of salmine, there are fairly wide differences in the estimated amounts of the other five amino acids.

Blackburn & Robson (1953) found that the ratio of isoleucine to valine in salmine hydrolysates was almost exactly 1:3; a finding which agrees fairly well with that of Block *et al.* (1949), but not with the other analyses. We have, therefore, carried out a full analysis of our salmine sulphate sample to see if its amino acid composition is significantly different from that of samples hitherto analysed.

EXPERIMENTAL

Chromatographic techniques

The amino acid separations were carried out by chromatography on starch columns 30 cm. in length and 0·9 cm. in diameter by the methods of Stein & Moore (1948). Two samples of starch were used, one being manufactured by Gordon Slater Ltd., Manchester, and the other (labelled Jalan A48) supplied by James Laing & Co. Ltd., Manchester. Both samples gave satisfactory resolution of the amino acids but the Jalan A48 starch was the finer of the two, a 30 cm. column having a flow rate of 1·3 ml./hr. under 15 cm. Hg pressure, whilst the Gordon Slater starch gave a flow rate of 1·5 ml./hr. under a pressure of 6 cm. Hg. The Jalan A48 starch, however, had a much higher column blank than the Gordon Slater starch, whose blank was equal to that of the developing solvent.

During early work with starch chromatograms it became apparent that the optimum load of 3 mg. of amino acids quoted by Stein & Moore (1948) was not sufficient to afford accurate analyses of isoleucine and alanine in salmine as they each only constitute about 1% of the protamine. It was found, in common with Stein & Moore, that loads greater than 3 mg. of amino acids caused flattening and broadening of the peaks, and prevented separation of proline from alanine and of serine from glycine. By modifying the method of applying the amino acid mixture to the column, however, we found that we could increase the load up to 8 mg. of amino acids without impairing the resolution of isoleucine, valine, proline and alanine. A suitable portion of amino acid mixture or protein hydrolysate was delivered from a microburette on to a circle of Whatman no. 3 filter paper (0·9 cm. diam.) and dried in a stream of warm air. After the surface of the starch column had been carefully dried and tamped, the paper circle was dropped on to it and pressed gently into place with a stainless-steel rod. 0·5 ml. of developing solvent was run over the end of the rod, allowed to drain on to the column surface, and driven in under pressure. A further two 0·5 ml. amounts of solvent were similarly added. The column was then prepared for development and transferred to the fraction cutter, which was similar in design to that used by Stein & Moore (1948).

Three different types of chromatograms were employed in the analysis of salmine hydrolysates. Accurate estimations of isoleucine, valine, proline and alanine were obtained using columns loaded with 8 mg. of salmine hydrolysate, and developed with a mixture of *n*-butanol, *n*-propanol and 0·1 N-HCl (1:2:1, v/v) (solvent 1). 0·5 ml. fractions were collected. Serine and glycine could not be satisfactorily resolved with such a heavy load, and so proline, valine, serine and glycine were separated on columns loaded with 4 mg. of hydrolysate and developed with solvent 1, 1 ml. fractions being collected. Finally, serine, glycine and arginine were separated on columns loaded with 2 mg. of hydrolysate and developed with solvent 1 until 36 ml. of solvent had emerged, when the solvent was changed to a mixture of *n*-propanol and 0·5 N-HCl (2:1, v/v) (solvent 2). 0·5 ml. fractions were collected. This method of analysing salmine, besides giving good resolution and optimum accuracy of estimation of the amino acids, enabled two chromatograms per week to be analysed. Two chromatography tubes of identical dimensions were used.

Amino-acid estimations

The amino-acid contents of all the fractions collected were estimated by a modification of the ^{64}Cu radiometric method of Blackburn & Robson (1953). The three types of fraction were treated as follows: (1) 0·5 ml. fractions of solvent 1 were neutralized and buffered by the addition of 2·5 ml. of a solution made up from 4 vol. of 0·18 M-Na_2HPO_4 and 1 vol. of 0·025 N-NaOH, and 1 ml. of radioactive copper phosphate suspension was added. (2) 3 ml. of a solution made up from 2 vol. of 0·27 M-Na_2HPO_4 and 1 vol. of 0·025 N-NaOH were added to 1·0 ml. fractions of solvent 1, followed by 2 ml. of radioactive copper phosphate suspension. (3) 0·5 ml. fractions of solvent 2 were neutralized and buffered by the addition of 2·5 ml. of a solution made up from 4 vol. of 0·18 M-Na_2HPO_4 and 1 vol. of 0·167 N-NaOH, and 1 ml. of radioactive copper phosphate suspension was added. The above mixtures gave only one liquid phase; they were thoroughly shaken and allowed to stand for 30 min. The subsequent filtration of the solutions and radiometric assay of the filtrates were carried out as described by Blackburn & Robson (1953).

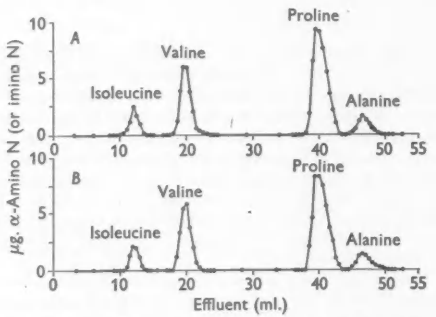

Fig. 1. *A*: separation of isoleucine, valine, proline and alanine from a salmine sulphate hydrolysate on a 30 × 0·9 cm. starch chromatogram developing with solvent 1. Amino N (or imino N) determined radiometrically. Total amino-acid load about 8 mg.; 0·5 ml. fractions. *B*: separation of DL-isoleucine, DL-valine, DL-proline and DL-alanine from a mixture of DL-isoleucine, DL-alanine, DL-valine, glycine, DL-proline, DL-serine and L-arginine made up in the molecular ratios

1:1:3:4:6:6:48,

under the same chromatographic conditions as *A* above.

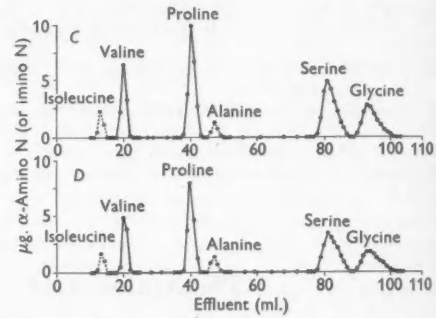

Fig. 2. *C*: separation of isoleucine, valine, proline, alanine, serine, and glycine from a salmine sulphate hydrolysate on a 30 × 0·9 cm. starch chromatogram developing with solvent 1. Amino N (or imino N) determined radiometrically. Total amino-acid load about 4 mg.; 1·0 ml. fractions. *D*: separation of DL-isoleucine, DL-valine, DL-proline, DL-alanine, DL-serine and glycine from the synthetic amino acid mixture used for *B* (Fig. 1) under the same chromatographic conditions as *C* above. Total amino-acid load about 3 mg.

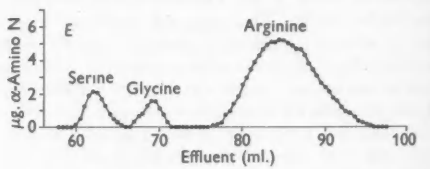

Fig. 3. *E*: separation of serine, glycine and arginine from a salmine sulphate hydrolysate on a 30 × 0·9 cm. starch chromatogram, changing developer after 36 ml. from solvent 1 to solvent 2. Amino N determined radiometrically. Total amino-acid load about 2 mg.; 0·5 ml. fractions.

The activities, in counts/min., of the fractions were plotted against their fraction numbers to give curves similar to those shown in Figs. 1–3. The average of four blank counts was determined, both before and after an amino acid peak where possible, and subtracted from the counts of those fractions contributing to the peak. The counts remaining, due to the copper-amino acid complex, were summed, and divided by the counts/min./μg. of α-amino N (or imino N) for the amino acid being estimated, to give the total amount of α-amino N in the amino acid peak. The counts/min./μg. of α-amino N (or imino N) for the amino acids estimated were derived from the mean difference in counts between four solutions containing 10 μg. of α-amino N and four solution blanks. The reaction conditions for these solutions were the

same as those for the column eluent fractions except for the small amount of NaCl formed by neutralization of the HCl in these fractions with NaOH, which was omitted. Since both column blanks and 'standard blanks' gave the same counts when Gordon Slater starch was used for the columns, the effect of the NaCl was considered to be negligible.

The samples of glycine, DL-alanine, DL-valine, DL-proline, DL-serine, DL-isoleucine and L-arginine used as standards were tested by filter-paper chromatography and elementary analysis, and shown to be free from amino acid impurities and from ammonia, sulphate and halide ions.

Decomposition of the amino acids of salmine during hydrolysis

Rees (1946) has shown that serious losses of serine and threonine occur during the hydrolysis of proteins and, since serine is one of the constituent amino acids of salmine, experiments were carried out to determine the amounts of this and of any other amino acids lost during the hydrolysis of salmine.

0·2 g. of glycine, DL-alanine, DL-valine, DL-isoleucine, DL-serine, DL-proline and L-arginine were refluxed separately with 20 ml. of 5·5N-HCl for 24 hr., the HCl removed by vacuum distillation and the residues dissolved in 50 ml. of water. Suitable samples were taken from the solutions and their NH_3 contents determined with the Markham (1942) apparatus, using a suspension of $Mg(OH)_2$ to liberate the NH_3. The amino-acid contents of other samples from the solutions were determined radiometrically by the ^{64}Cu method of Blackburn & Robson (1953). Only serine and arginine decomposed under acid reflux, and the losses of these amino acids during the boiling with 5·5N-HCl of amino-acid mixtures made up to simulate salmine hydrolysates were estimated. The NH_3 liberated during the hydrolysis of five 1 g. amounts of salmine sulphate with 20 ml. of 5·5N-HCl for 24 hr. was determined. No NH_3 was liberated from salmine sulphate itself on boiling with $Mg(OH)_2$ suspension, nor from any of the amino acids used in these experiments.

Total N

The total N content of salmine sulphate was determined on an air-dried sample (Chibnall, Rees & Williams, 1943). Suitable amounts of the same sample were weighed out at the same time for estimations of ash, moisture, and SO_4^{2-} contents. The Kjeldahl procedure employed was that recommended by Chibnall *et al.* except that Se powder was substituted for sodium selenate. Digestion was for 16 hr. Six determinations were made.

Ash

A weighed sample of air-dried salmine sulphate was ashed at 800° in a Pt crucible to constant weight. This gave the 'non-sulphated ash' weight. The ash was then treated with small successive amounts of H_2SO_4 and reheated to constant weight. This gave the weight of 'sulphated ash'.

Moisture

A weighed sample of air-dried salmine sulphate was heated to constant weight in an oven at 102°.

Sulphate

The SO_4^{2-} content of an air-dried sample of salmine sulphate was determined gravimetrically as $BaSO_4$ by double decomposition with excess $BaCl_2$ in water. Analyses were carried out in triplicate.

Material and hydrolysis procedure

The salmine sulphate used was supplied by Boots Pure Drug Co. Ltd., and had been prepared from the same batch of salmon milt as the sample previously analysed by Blackburn & Robson (1953). Hydrolysates for amino acid analysis and NH_3 determinations were prepared by refluxing 1 g. samples with 15 ml. of 6N-HCl for 24 hr. HCl was removed *in vacuo* and the residue made up to 10 ml. with water.

RESULTS

A typical set of results obtained for the estimation of proline separated from a mixture of DL-isoleucine, DL-valine, DL-proline, DL-alanine, DL-serine, glycine and L-arginine, made up in the molecular ratios 1:3:6:1:6:4:48, is shown in Table 1. The amino acid mixture was made up to simulate a salmine hydrolysate as closely as possible. The estimation of proline is worked out in full from the data given in order to clarify the procedure adopted. The proline peak obtained from the data is that plotted in graph *B* (Fig. 1), together with the peaks due to isoleucine, valine and alanine. In graph *A* are plotted the peaks of the same four amino acids separated from a salmine sulphate hydrolysate under the same chromatographic conditions, and the close similarity of the two curves is obvious. Similarly, graphs *C* and *D* (Fig. 2) illustrate the parallelism which exists between the isoleucine, valine, alanine, proline, serine and glycine peaks of the hydrolysate and of the synthetic amino-acid mixture. Although the fractions comprising the isoleucine and alanine peaks were analysed in order to plot the curves, the results obtained were not sufficiently accurate to be used for the estimation of these amino acids, and the peaks are therefore drawn as dotted lines. Graph *E* (Fig. 3) shows the resolution of the serine, glycine and arginine peaks.

Analyses of the seven amino acids separated from the synthetic mixture gave recoveries within the limits $100 \pm 3\%$ for DL-isoleucine, DL-serine, DL-alanine, glycine and L-arginine and $100 \pm 2\%$ for DL-valine and DL-proline. These recoveries compare favourably with those obtained by Stein & Moore (1948). Two salmine sulphate hydrolysates were analysed and the full results from the first hydrolysate are given in Table 2 together with mean values for five amino acids from the second hydrolysate. Although the alanine and glycine contents of this hydrolysate were not determined, we considered that the estimations for these five amino acids were sufficiently in accord with those obtained from the first hydrolysate to make further analyses unnecessary.

When the seven constituent amino acids of salmine were refluxed separately for 24 hr. with

Table 1. *Analysis of proline fractions separated from a* 1:4:6:1:6:4:48 *(mol. prop.) mixture of* DL-*isoleucine,* DL-*valine,* DL-*proline,* DL-*alanine,* DL-*serine, glycine and* L-*arginine on starch chromatogram developed with solvent* 1. *(See Fig.* 1, *B)*

(The calculation of the amount of proline is as follows: activity of 1 μg. of imino N (proline standard) = 443·0–74·6 = 368·4 counts/min., therefore imino N in fractions 77–86 is 16 730/368·4 = 45·4 μg.; imino N (proline) applied to chromatogram = 44·8 μg. Recovery, 101·4 %.)

Activities of proline standards and blanks		Starch chromatogram no. 23 proline fractions			
Imino N (proline) (μg.)	Activity (counts/min.)	Fraction no.	Activity (counts/min.)	Counts/min. (less average blank)	Amount of proline (μg. imino N)
10·0 (1)	4460	73*	768	24	—
10·0 (2)	4420	74*	732	−12	—
10·0 (3)	4440	75*	739	−5	—
10·0 (4)	4400	76*	749	5	—
Average	4430	77	1595	851	2·32
		78	2502	1758	4·77
Blanks		79	3803	3059	8·32
(1)	727	80	3819	3075	8·35
(2)	757	81	3506	2762	7·53
(3)	752	82	2894	2150	5·85
(4)	748	83	2162	1418	3·85
		84	1743	999	2·72
		85	1183	439	1·18
Average	746	86	963	219	0·60
		87*	749	5	—
		88*	761	17	—
		89*	708	−36	—

* Average column blank (tubes 73–76 and 87–89 inclusive) = 744 counts/min.

Table 2. *Analyses of the amino acids in a salmine sulphate hydrolysate*

Starch chromatogram no.	(g. amino acid/100 g. salmine) (see text)						
	Isoleucine	Alanine	Valine	Glycine	Serine	Proline	Arginine
9	1·30	0·87	3·72	—	—	6·95	—
12	1·27	0·88	3·65	—	—	6·80	—
13	1·27	0·91	3·70	—	—	6·93	—
14	—	—	3·66	3·12	6·19	6·94	—
16	—	—	3·66	2·95	6·19	6·90	—
17	Unfinished chromatogram				6·40	—	—
18	—	—	—	3·01	6·31	—	85·8
20	—	—	—	2·98	6·33	—	87·5
21	Arginine only determined						86·0
Mean values	1·28	0·89	3·67	3·01	6·28	6·90	86·4
Mean values for second hydrolysate	1·27	—	3·68	—	6·28	6·87	86·5

5·5 N-hydrochloric acid only serine and arginine decomposed to give ammonia. Radiometric analyses of the residual amounts of all seven amino acids after refluxing showed that only serine decomposed by more than 2 %. The percentage loss of serine as measured by the ammonia liberated was found to be 8·9 %, and as measured by the radiometric copper method 9 ± 1 %. The ammonia liberated by arginine corresponded to a loss of 0·31 %, assuming 1 mol. of arginine to give 1 mol. of ammonia on decomposition, and this loss could not be detected by the radiometric assay. When mixtures of glycine, alanine, valine, isoleucine, serine, proline and arginine in the molecular ratios of (*a*) 4:1:3:1:0:6:50 and (*b*) 4:1:3:1:7:6:50 were refluxed for 24 hr. with 5·5 N-hydrochloric acid the average losses of serine and arginine, as measured by the ammonia liberated, were 8·7 and 0·30 % respectively. It was assumed that the loss of arginine in mixture (*b*) was the same as that in

mixture (*a*), i.e. 0·30 %. Mixture (*a*) was made up to simulate salmine less its serine, and mixture (*b*) the complete salmine.

The mean of five determinations for the amount of ammonia liberated during hydrolysis of salmine sulphate was 0·204 ± 0·003 g./100 g. salmine, the lowest and highest amounts observed being 0·193 and 0·218 g./100 g. salmine respectively. The amount of ammonia expected from the serine, assuming that it decomposes in the protamine at the same rate as it does in the free state, is 0·0904 g. and that from arginine 0·0262 g., giving a total of 0·117 g./100 g. salmine. From these figures it seems clear that either serine or arginine (or both) decompose at a more rapid rate when combined in the protamine than they do in the free state. Rees (1946) refluxed salmine with 20 % hydrochloric acid for 24 hr. and found the liberated ammonia to be 0·15 g./100 g. salmine (figure calc. from Table 6 of his paper). Using his correction factor of 100/89·5 for serine he states that the whole of this ammonia can be accounted for as being due to serine decomposition. However, he has made it clear that for all the proteins he analysed his correction factors for serine and threonine cannot account for all the ammonia found after hydrolysis, and states that this may be due to serine and threonine decomposing more rapidly when combined in the protein than in the free state. In arriving at our correction factors for serine and arginine decomposition during the hydrolysis of salmine sulphate we have assumed that the ratio of the rates of decomposition of the two amino acids when combined are the same as in the free state. Whether this is in fact the case it is impossible to say, but it is the only reasonable assumption that can be made. On this basis the fraction of ammonia found in salmine hydrolysates due to decomposition of serine is 0·164 g./100 g. salmine (80 % of the total), equivalent to a serine loss of 1·01 g./100 g. salmine, and corresponding to a correction factor of 100/86. Hence the observed serine amount of 6·28 g./100 g. salmine is corrected to 7·29 g./100 g. salmine. The corresponding correction factor of 100/99·5 for arginine has not been applied since the arginine assay is not sufficiently accurate to warrant its use.

The total nitrogen content of moisture-free salmine sulphate was 24·3 %, and its sulphate content 20·5 %. The ash content was 1·55 % and the 'sulphated' ash content 1·70 %. A semiquantitative analysis of the 'non-sulphated' ash showed that it contained about 15 % of calcium, 35 % of sulphate, and appreciable amounts of phosphate. Allowing for the percentage of 'non-sulphated' ash and for the sulphate content of salmine sulphate, we assumed the total nitrogen content of free salmine to be 31·1 %, and the above data are reported in terms of this hypothetical free base.

DISCUSSION

The analytical results obtained in the present work show that, within the limits of the methods used (± 3 %) the amino acids isoleucine, alanine, valine, glycine, proline, serine and arginine occur in salmine in the molecular ratios shown in Table 3, and that the minimum molecular weight of the protamine, if it is homogeneous, is about 10 000.

We have pointed out in the introduction to this paper that the analyses of previous workers show fairly good agreement for the amounts of arginine and glycine in salmine; our own analyses are in accord with their figures (Table 4). Estimates of the other five amino acids do not show good overall agreement, although there may be agreement between two or more sets of authors for any particular amino acid. These differences become more apparent when the estimated minimum molecular weight for salmine, and the number of residues of each of its constituent amino acids, are calculated from the amino-acid analyses. From his analysis of the protamine, Tristram (1947) gave the minimum molecular weight as 8000 and found that the ratios of isoleucine, alanine, valine, glycine, proline, serine and arginine were 1:1:2:3:4:7:40, but Velick & Udenfriend (1951) found that alanine, valine, glycine, proline, serine and arginine occur in the ratios 1:3:3:4:4:34 and gave a value of 7000 for the minimum molecular weight. The last-named authors have pointed out that, whilst the analytical discrepancies may be due to differences in methods of analysis it is also possible that they may be

Table 3. *Molecular proportions of amino acids of salmine*

Amino acid	Amino acid N as % total N	Amino acid/ 100 g. salmine	Minimum mol.wt.	No. of residues	Calculated mol.wt.
Isoleucine	0·44	1·28	10 240	1	10 240
Alanine	0·45	0·89	10 000	1	10 000
Valine	1·40	3·68	3 190	3	9 570
Glycine	1·80	3·01	2 500	4	10 000
Serine	3·12	7·29	1 440	7	10 080
Proline	2·70	6·90	1 670	6	10 020
Arginine	89·80	86·40	202	50	10 100
Total	99·71	109·44		72	

Table 4. *Amino acid analyses of salmine by various authors*

	N as % total N of protamine								
					Velick & Udenfriend (1951)				
Amino acid	Mills (1952)	Tristram (1947)	Hamer & Woodhouse (1949)	Block *et al.* (1949)	Micro-biological*	Isotope	Ion-exchange	Blackburn & Robson (1953)	Present work
Arginine	90·6	89·0	91·0	90·4	—	—	86·3	90·6	89·8
Isoleucine	1·85	0·56	0·62	0·41	—	—	—	0·45	0·44
Valine		1·20	1·47	1·56	1·65	1·92	1·81	1·44	1·40
Glycine	1·97	1·78	2·17	1·96	—	1·89	1·90	—	1·80
Alanine	0·59	0·56	0·30	0·75	—	0·58	0·55	—	0·45
Proline	2·16	2·29	1·96	3·06 to 3·33	2·50	2·62	2·70	—	2·70
Serine	2·70	3·94	2·41	2·96	3·64	2·74	2·70	—	3·12

* Microbiological assays carried out by Dr G. B. Ramasarma.

accounted for by differences in origin of the salmine samples. They claim, however, that the salmine used seemed to be homogeneous from the data obtained from its sedimentation and electrophoretic mobility; that, whilst salmine may be a family of closely related peptides, this is not very probable in view of the fact that all the amino ends of the chains terminate in proline and that the ratio of mono-amino acids to arginine cannot be a structural variant since the protamine is electrically homogeneous. Recently, however, Felix (1952) has stated that there is good evidence that protamines are not homogeneous, but consist of several components with very similar composition but different molecular size, ranging probably from 2000 to 10 000. He cites the case of clupeine from which about ten components were separated by means of countercurrent distribution. The data on the amino acid composition of salmine obtained in the present work do not prove conclusively that the salmine sample analysed was homogeneous, but the fact that the amino acid residues occur in integral ratios within the limits of experimental error is evidence in favour of homogeneity.

SUMMARY

1. A complete amino acid analysis of a specimen of salmine sulphate has been made.

2. The losses of serine and arginine during the acid hydrolysis of salmine sulphate have been experimentally assessed; the serine loss was estimated to be 14 % and the arginine loss 0·5 %.

3. The amino acids isoleucine, alanine, valine, glycine, proline, serine and arginine were found to occur in salmine in the molecular proportions 1:1:3:4:6:7:50. On this basis the minimum molecular weight of salmine was calculated to be about 10 000 if the protamine is a single substance.

4. The problem as to whether salmine may be regarded as a single substance has been discussed briefly in the light of available analytical data.

We are indebted to Miss B. Skinner for considerable assistance with the experimental work, and to Messrs R. Gibson, S. Dilworth and G. H. Hines for the design and construction of the fraction cutter. Our thanks are also due to Dr A. J. P. Martin, F.R.S., and Dr F. O. Howitt for helpful advice and criticism during the course of this work, and to the Director and Council of the Wool Industries Research Association for permission to publish this paper.

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Oat Lipase

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Since the initial observations of the action of pancreatic lipase on neutral fat by Eberle in 1834 (see Sumner & Somers, 1943), lipases from various sources have been the subject of much detailed investigation. Most of this work has been directed at the elucidation of the mode of action of animal lipases, pancreatic lipase in particular, because of their obvious importance in the digestion and absorption of fat by the animal body. Among the plant lipases, that of the castor bean (*Ricinus*) has received most attention owing primarily to its relatively high activity. More recently, Fiore & Nord (1950) have published a comprehensive study on the lipase of *Fusarium lini*.

Cereal lipases have received relatively little attention. It was shown by Maestrini (1919, 1921*a*, *b*) that germinated barley and by Van Lear (1921) that malt extracts had lipase activity. Sullivan & Howe (1933) and Kretovitch, Sokolova & Ushakova (1940) have studied the lipase activity of wheats. More recently, Singer & Hofstee (1948) have made detailed studies on wheat-germ lipase.

In the present investigation oat lipase has been obtained in a highly purified condition relative to its activity in an oatmeal and the purified enzyme has been shown to split off, specifically, a single butyric acid radical from tributyrin. The latter observation has already been published in a preliminary note (Peers, 1953). Oat lipase is located in the outer pericarp layers of the groat and it is possible to remove this lipase from the groats by a 'wet-scrubbing' technique (Hutchinson, Martin & Moran, 1951). This paper is a more complete account of the results obtained in a general study of oat lipase, which appears to differ in certain properties from lipases from other sources.

METHODS

Wet-scrubbing technique for removing oat pericarp. The apparatus (see Fig. 1) consists essentially of a test-tube brush (3 in. × 0·75 in.) mounted in the chuck of a stirrer motor ($\frac{1}{20}$ h.p.) revolving in a container made of two Quickfit adaptors (MF 18/5 and DA 59). The side arm of the lower adaptor contains a copper spiral (*C*) which retains the groats but allows the particles of pericarp in suspension to pass through a rubber tube which can be connected to a Büchner flask. The process is a 'batch' one, 10 g. of groats being scrubbed at a time in a total of 50 ml. of distilled water. The groats are placed in the container, 25 ml. of glass-distilled water added with the tube in position *A* and the brush revolved at approx. 2000 rev./min. for 2 min. Near the end of this period the tube is inserted in the Büchner flask and the liquor removed by suction (position *B*). With tube again in position *A* a further 25 ml. of water are added and scrubbing continued for a further 1 min. and then the liquor is drawn off. Approx. 95% of the water added is recovered as the liquor. The apparatus is conveniently cleaned out ready for the next batch by removing *S* and, with the brush slowly revolving, water is poured into the apparatus.

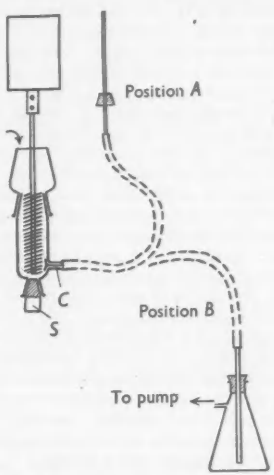

Fig. 1. Apparatus for scrubbing groats. For explanation see text.

Dough method for measurement of lipase activity. A modification of the method described by Hutchinson & Martin (1952) was used. Whole groats were inactivated with respect to lipase activity by immersion, in a sealed tube, in a boiling-water bath for 1 hr. The groats were then ground in a high-speed Christy-Norris disintegrator mill to pass through a sieve of aperture size 0·7 mm. The meal was defatted by two successive washings with light petroleum (40–60°) using approx. 50 ml./10 g., the solids being separated by centrifugation each time. The inactive, defatted oatmeal was spread out in a thin layer on filter paper and dried by exposure to the air. Samples (0·5 g.) were weighed into small test tubes (2 × 0·5 in.) and eight drops of olive oil,

equivalent to 149±6 mg. (B.P. grade: free fatty acid content <1%) were added from a standard pipette and mixed thoroughly with a glass rod. Water (0·2 ml.) or the solution (or suspension) under test was added at zero time and the mixture quickly worked into a dough with the glass rod. The tube was corked and immersed in a water bath at 37°. After an appropriate time interval the contents of the tube were washed into a 50 ml. centrifuge tube with light petroleum (40–60°) and after centrifuging at 3000 rev./min. for 15 min. the supernatant was carefully decanted into a tared 100 ml. extraction flask. The petroleum was distilled off, the flask dried in a vacuum desiccator over silica gel and then weighed to check the recovery of oil and fatty acid. The results were rejected if the net weight was outside the limits 149±6 mg.

Approximately 30 ml. of neutralized, boiling benzene: ethanol (1:1, v/v) were added and the sample titrated, whilst boiling on a hot-plate, with ethanolic 0·0354 N-NaOH (1 ml. = 10 mg. free oleic acid). Olive oil is composed of mixed triglycerides with oleic acid as the predominant fatty acid (Hilditch, 1947). Calculations based upon the published constitution of olive oil show that the errors introduced by regarding it, for the purposes of lipase assay, as triolein, are negligible.

The lipase activity (q_{oleic}) is expressed as m-moles oleic acid released/hr./g. dry wt., and under these conditions 1 unit of lipase activity releases 1 m-mole oleic acid in 1 hr.

Manometric assay of lipase activity. This was based on the method of Rona & Lasnitski (1924). The activity of the lipase preparations was measured against tributyrin in the conventional Warburg apparatus at 37° using a bicarbonate: CO_2 buffer system at pH 7·4. Each flask contained 1·5 ml. 0·0246 M-$NaHCO_3$, 0·5 ml. of a dispersion of tributyrin (4·0%, w/v) in 0·0148 M-$NaHCO_3$ (in side bulb) and 1 ml. of enzyme solution or suspension. The manometers were gassed with 5% CO_2 + 95% N_2 and equilibrated for 10 min. in the bath at 37° prior to tipping the substrate. The manometers were shaken at 150 oscillations/min. The CO_2 evolution was measured over 30 min. and the rate of release of CO_2 over the initial 10 min. period (corrected for by appropriate blanks) was calculated. The activity ($q_{butyric}$) is expressed as m-moles CO_2 released/hr./g. dry wt.

Saponification values. These were determined on a semi-micro scale in boiling tubes fitted with reflux air condensers. Samples (100–150 mg.) of the glyceride were refluxed on a water bath with 10 ml. ethanolic 0·2 N-KOH for 45 min. The tubes were then cooled, phenolphthalein added and the excess KOH back-titrated with 0·1 N-HCl.

Nitrogen determinations. Determinations of total N were carried out in a Kjeldahl apparatus after 5 hr. digestion by the procedure described by Hiller, Plazin & Van Slyke (1948).

Phosphorous determinations. These were carried out according to Allen (1940). The colour densities were read on a Hilger 'Spekker' absorptiometer against a reagent blank using spectrum red (H 508) and heat-resistant filters. The amidol-bisulphite reagent was made up fresh daily and filtered before use.

RESULTS

Evidence that the lipase is a true oat lipase

As the lipase activity of oats is found mainly in the epidermal layers it is important to know that it is essentially a property of the seeds, and does not arise from subepidermal fungal mycelium, which is known to occur in some specimens of grain.

Observations on the subepidermal fungal contaminants of wheat grains made by Oxley & Jones (1944), Hyde (1950) and Hyde & Galleymore (1951) suggested that fungus-free oat grains might possibly be obtained by keeping them in a dry atmosphere for 3–4 weeks prior to harvesting. This was tested, with selected oat plants, by clamping a 3 kg. jar containing bags of silica gel over the heads, the stalks passing through a split cork. Control heads were grown in the same plot. The silica gel was regenerated every 2 days. In this way samples were obtained which varied in mycelial content and lipase activity. The mycelial content was determined by removing some epidermis from externally 'sterilized' groats and staining with aniline blue as described by Hyde & Galleymore (1951). These results are shown in Table 1.

Oats examined while at the 'milky' stage (i.e. at an early stage in the development of the grain) were free of mycelium but high in lipase activity.

Pieces of pericarp from groats which had been treated to sterilize the external surface, but which contained subepidermal mycelia, were plated out on 2% malt agar and incubated at 25° for 7 days. Such cultures indicated that two principal subepidermal contaminants of groats were an *Alternaria* species and an *Aspergillus* species. These two fungi, isolated from the groats, were grown on 2% malt agar at 25°, the mycelia were harvested after 4 days and their lipase activities determined by the dough method on a 'homogenized' suspension of the mycelium. The activities (m-moles oleic acid/hr./g. dry wt.) were 0·13 for the *Alternaria* mycelium and 0·19 for the

Table 1. *Lipase activity and sub-epidermal fungal contaminants in oats*

(The oat heads were kept in an atmosphere dried with silica-gel for 1 month prior to harvest; control oats were grown for each sample. The lipase activities were determined against olive oil.)

Sample	Conditions of growth	$q_{oleic\ acid}$ (m-moles/hr./g. dry wt.)	Mycelial content
A	(1) Grown in dry atmosphere	0·65	Nil
	(2) Control	0·42	+ + + +
B	(1) Grown in dry atmosphere	0·54	+
	(2) Control	0·31	+ + +

Aspergillus mycelium, compared with 0·31 for the whole oatmeal. Since the mycelial content of the groats constituted approximately 2 % by weight of the pericarp which in turn constituted approximately 2 % by weight of the whole groat it follows that the mycelial contamination could not possibly account for the total lipase activity.

Distribution of lipase activity in fractions obtained by wet-scrubbing Victory groats

The groats were scrubbed as described, the liquor centrifuged at 3000 rev./min. for 30 min. to separate the pericarp which had been scrubbed off, and the pericarp and the depericarped groats were spread out on filter paper and air dried. The three fractions were assayed for lipase activity and analysed for dry matter, nitrogen and phosphorus. Table 2 summarizes the results obtained.

Purification and some properties of oat lipase

The data given in Table 2 show that the lipase in the centrifuged extract obtained from scrubbed groats was considerably purified (approximately 300-fold on dry-matter basis: 350-fold on a nitrogen basis). The centrifuged aqueous extract was somewhat opalescent and could only be clarified by repeated filtration through the same filter paper. The clear, filtered extract had very little lipase activity and the activity was associated with the material causing the opalescence.

Precipitation occurred when the pH of the opalescent extract was adjusted to 5·5 with acetic acid with further precipitation at pH 4·8. Some lipase activity was found in the former fraction but analysis showed that over 80 % of the lipase had been inactivated by this procedure. Ethanol added up to a concentration of 75 % (v/v) to the cooled aqueous extract did not produce a precipitate. With ammonium sulphate added up to 80 % saturation a precipitate settled out after 30 min. at 0–4°, but both the precipitate and the supernatant were inactive. Both precipitate and supernatant were dialysed and retested separately and combined but no activity could be observed. The original extract after dialysis overnight against distilled water showed a 20 % reduction in activity, and with saturated ammonium sulphate added up to a concentration of 50 % saturation showed a 50 % loss in activity, although no precipitation occurred.

Lipase was adsorbed, from aqueous extracts, by calcium phosphate gel (prepared according to Singer & Kearney, 1950), kieselguhr and alumina Cγ, but it was not possible to elute the lipase bound to these adsorbents although the adsorbent-lipase complex itself was lipolytically active. The calcium phosphate gel-lipase complex retained its activity for 10 days when stored at 0–4°. Extraction of the complex with *iso*butanol:water (1:1) caused the activity to disappear from the solid phase but no activity could be found in the water phase, in the *iso*butanol phase, in the slight interfacial precipitate which formed at the junction of the two liquid phases or in any combination of these fractions.

By repeating a cycle of freezing, and then thawing at about 17°, a precipitate formed which contained most of the original lipase activity. This precipitate was insoluble in a wide range of solvents including the ether:petroleum mixtures of Takamiya (1935) used for *Ricinus* lipase, the chloroform:methanol system of Folch & Lees (1951) used for proteolipids, and the α-picoline-dioxan system of Takashima (1952) used for lipoprotein-chlorophyll complexes.

This complete insolubility of the enzyme after the freezing-thawing treatment ruled out the usual techniques for further purification, and accordingly the possibility was examined of removing inert material before preparing the opalescent extract. Extraction of the groats with organic solvents prior to scrubbing removed some inert material and so a continuous cold extractor was constructed of Quickfit apparatus in which the groats, contained in a Soxhlet thimble, were continuously extracted with a solvent for approximately 8 hr. A series of solvents were tried and after aerating off the solvent, 10 g. samples of the groats were scrubbed in 50 ml. of water and the centrifuged aqueous extracts examined for lipase activity. No loss of activity from the groats occurred with petroleum ether (40–60°) or benzene and ether, a partial loss occurred with acetone, *n*-butanol and *iso*butanol and complete loss with methanol, ethanol and chloroform: the ratio $q_{oleic}/q_{butyric}$ was between 2·45

Table 2. *Lipase activity in the various fractions obtained by scrubbing oats*

(The hulled oats (11·9 g.) were scrubbed in distilled water (100 ml.) and the suspension of pericarp centrifuged. All solid samples were air-dried to approximately 10 % moisture.)

Fraction	Amount	Total solids (g.)	Total N (mg.)	Total P (mg.)	Lipase activity	
					q_{oleic} (m-moles/hr./g. dry wt.)	$q_{butyric}$ (m-moles/hr./g. dry wt.)
Wholemeal	11·86 g.	10·20	258·7	42·15	0·77	0·31
Depericarped groats	11·42 g.	9·89	253·4	41·21	0·05	0·02
Pericarp	0·22 g.	0·21	3·3	0·73	3·65	1·53
Aqueous extract	94 ml.	0·04	0·9	0·17	234	94·50

and 2·56 in all cases. Ether was found to give the cleanest extract after centrifuging, and the following technique was finally adopted for the preparation of the partially purified oat lipase.

Groats which had been pre-extracted with ether were 'scrubbed' and the aqueous extract collected as described in the Methods section. This extract was centrifuged at 4500 rev./min. for 30 min. and the slightly opalescent solution so obtained frozen and thawed several times until precipitation had occurred. The suspension was then centrifuged and the precipitate resuspended in distilled water and washed several times; it was found necessary to freeze and thaw to avoid losses in the washing process. The material so obtained is the purified lipase which has been used for a study of some of its properties. The purification is some 2000-fold on a dry-matter basis compared with the activity in an oatmeal prepared from the same groats.

Nitrogen and phosphorus contents have been determined during this purification process and these figures are included in Table 3 which shows the activities, etc., at each stage. A number of preparations made by this procedure have been found to have similar nitrogen and overall purification figures. The lipase prepared in this manner is stable over a period of 10 days when kept at 0–4°, pH 7·4.

Samples of the preparation have been acid-hydrolysed (6N-hydrochloric acid) and the hydrolysate examined on two-dimensional paper chromatograms using phenol : NH_3 and *n*-butanol : acetic acid as irrigating solvents and ninhydrin as indicator. These chromatograms show all the commonly occurring amino acids and ethanolamine.

The centrifuged aqueous extract was examined by paper electrophoresis, using the technique of Gordon, Gross, O'Connor & Pitt-Rivers (1952). This technique showed the presence of at least three protein components. Two of these remained in solution after the freezing and thawing technique and the third, which was the major protein component, did not migrate in 0·05M-veronal buffer, pH 8·5, or in 50% (v/v) ethanol containing 5% (w/v) 0·880 ammonia.

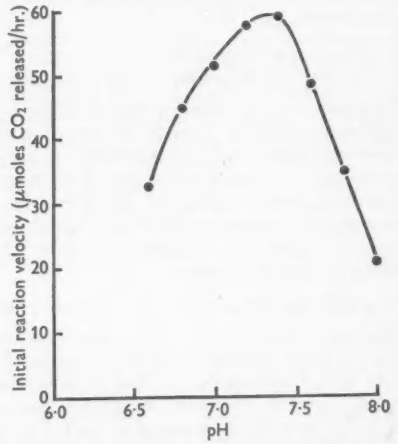

Fig. 2. Effect of pH on the activity of purified oat lipase. Initial reaction velocities (expressed as μmoles CO_2 released/hr.) are measured at 37° in $NaHCO_3$:CO_2 buffer systems in the presence of 20 mg. tributyrin and 0·21 purified lipase units/flask, total vol. 3 ml.

Some kinetic properties of the purified lipase

Effect of pH. Samples (1·0 ml.) of a suspension of the purified lipase (0·14 mg./ml. dry weight, 0·21 lipase units/ml.) were used in the manometric method to establish the pH-activity relationships at 37°. The manometer flasks contained 1·0 ml. enzyme, 0·5 ml. of a dispersion of tributyrin (4·0%, w/v) in appropriate strength sodium

Table 3. *Purification of oat lipase*

(20 g. of ether-extracted Victory groats were scrubbed in 100 ml. distilled water. The extract obtained was centrifuged at 4500 rev./min. for 30 min. and the supernatant obtained was alternatively frozen solid and thawed a total of six times. The precipitate formed was centrifuged off and washed with 3 × 10 ml. of distilled water. The solid samples were resuspended in water for assay purposes.)

Stage of preparation	Amount	Dry matter (mg./ml.)	N (mg./g. dry wt.)	P (mg./g. dry wt.)	Q_{oleic} (m-moles/hr./g. dry wt.)	$Q_{butyric}$ (m-moles/hr./g. dry wt.)	Purification on dry wt.	$\frac{Q_{oleic}}{Q_{butyric}}$
(1) Groats	20·0 g.	87·8	25·4	4·13	0·78	0·31	1	2·52
(2) Aqueous extract	98 ml.	3·78	19·2	4·15	47·6	19·3	61	2·47
(3) Centrifuged extract								
(*a*) Supernatant	97 ml.	0·60	36·8	7·93	281·0	114·0	361	2·47
(*b*) Residue	10 ml.	31·0	16·0	3·45	4·5	1·8	—	2·50
(4) Supernatant from (3) frozen and thawed								
(*a*) Supernatant	96 ml.	0·53	32·2	8·25	106	42·0	—	2·52
(*b*) Washed precipitate	10 ml.	0·70	66·0	5·20	1570	625	2000	2·51

bicarbonate and 1·5 ml. of sodium bicarbonate solutions varying from 0·0039M to 0·098M: the manometers were gassed with a nitrogen-carbon dioxide mixture (5%:95%) and the rates of release of carbon dioxide measured over the initial 10 min. period. The results are shown in Fig. 2.

Effect of enzyme concentration. Using the manometric technique with 0·0148M-sodium bicarbonate to maintain the pH at 7·4, varying amounts of purified lipase in suspension were assayed under the usual conditions. The results showed a linear response up to a rate of release of carbon dioxide of 45 μmoles/hr. measured over the first 10 min.; at higher enzyme concentrations the initial reaction velocity falls off.

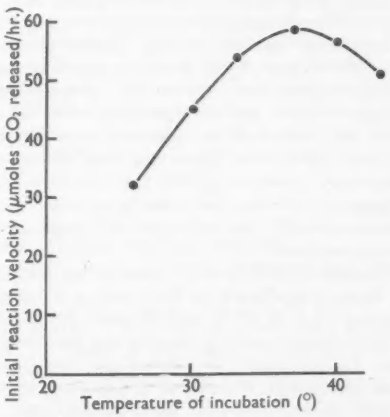

Fig. 3. Effect of temperature on the initial reaction velocity of purified lipase. Measured in 0·0148M-$NaHCO_3$ (pH 7·4) in the presence of 20 mg. tributyrin and 0·20 lipase units/flask, total vol. 3 ml.

Effect of temperature of incubation. Samples (1 ml.) of a purified lipase suspension (0·13 mg./ml. dry matter, 0·20 lipase units/ml.) were used to determine the initial reaction velocity against tributyrin over a range of incubation temperatures. The results obtained are shown in Fig. 4, and under these conditions indicate an optimum temperature of 37–38°.

Effect of substrate concentration. Using 1 ml. samples of the purified lipase (0·15 mg./ml. dry matter, 0·23 lipase unit per ml.) initial reaction velocities have been measured at initial tributyrin concentrations varying from $2{\cdot}2 \times 10^{-3}$ to 11×10^{-3}M. Reciprocal plots made as described by Lineweaver & Burk (1934) gave straight lines and from the intercepts on the axes the Michaelis constant (K_m) and the maximum initial reaction velocity (V) have been calculated as 0·0062M and 60·3 μmoles carbon dioxide/hr. (means of three determinations) respectively under these conditions.

Specificity and products of hydrolysis of tributyrin. It was noticed that the hydrolysis of tributyrin ceased when one-third of the total possible carbon dioxide had been released. Typical progress curves for two different amounts of tributyrin (3·33 and 5·0 mg. per flask) are given in Fig. 4. This suggested

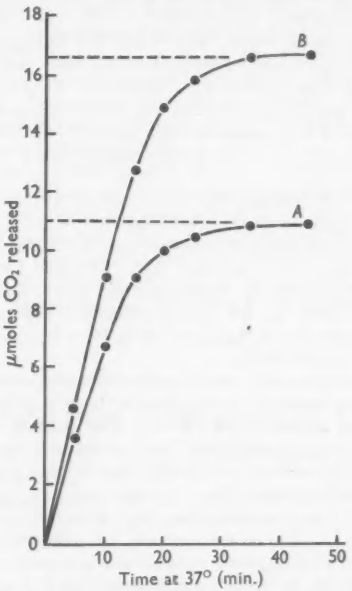

Fig. 4. Progress curves for purified oat lipase acting on tributyrin. Curves obtained using manometers (total vol. 3 ml.) containing 1·0 ml. lipase (0·13 mg./ml. dry matter: 0·20 lipase units/ml.), 0·0148M-$NaHCO_3$. The amounts of tributyrin were 11·0 μmoles (A) and 16·6 μmoles (B). The dotted lines show the theoretical one-third total possible CO_2 evolutions.

that only one of the butyric acid radicals was split off by the purified enzyme leaving a dibutyrin as a reaction product. Krebs's pots containing 60 ml. 0·0245M-sodium bicarbonate, 20 ml. of enzyme preparation (0·18 mg./ml. dry matter, 0·26 lipase units/ml.) 20 ml. of water and 20 ml. of 2% tributyrin dispersed in 0·0148M-sodium bicarbonate were incubated at 37° with shaking for 2 hr. after gassing with a nitrogen-carbon dioxide mixture (95%:5%). The contents of the pots were then transferred to a separating funnel and extracted twice with 50 ml. dry ether. The ethereal extract was evaporated on a water bath leaving a faintly yellow oil. In five experiments yields of 311·7, 306·6, 310·3, 307·8 and 306·2 mg. of this oil were obtained (theoretical yield of dibutyrin, 307 mg.).

These samples were bulked and fractionally microdistilled at 26 mm. vacuum, when approximately 95% of the oil distilled at 184–187°. The product gave a negative periodate-fuchsin reaction. (Found: C, 56·7; H, 8·5%; saponification value, 478. $C_{11}H_{20}O_5$ requires C, 56·9; H, 8·7%, saponification value, 483.)

α-Monobutyrin was prepared by the method of Schuette & Hale (1930) as a light-yellow oil, b.p. 140–142°/5 mm. (Found: C, 51·5; H, 8·9%; saponification value, 349. Calc. for $C_7H_{14}O_4$: C, 51·8; H, 8·7%; saponification value, 346.) β-Monobutyrin was prepared as described by Daubert (1940) as an oil, b.p. 141–143°/5 mm. (Found: C, 51·7; H, 8·7%; saponification value, 343. Calc. for $C_7H_{14}O_4$: C, 51·8; H, 8·7%; saponification value, 346.)

The two dibutyrins were prepared from the corresponding dibromohydrins according to the methods of Güth (1903) as oils, b.p. 169–172/20 mm. (αβ-), and 177–9°/20 mm. (αγ-). (Found: for αβ-: C, 56·8; H, 8·7%; saponification value, 481; for αγ-: C, 56·9; H, 8·7%; saponification value, 479; calculated for $C_{11}H_{20}O_5$; C, 56·9; H, 8·7%; saponification value, 483.)

These four compounds, and the isolated product of reaction, were not hydrolysed by freshly prepared, purified lipase at pH 7·4, 37°. The purified lipase preparation hydrolysed the water-soluble triglyceride, triacetin, at roughly one-third of the rate it attacks tributyrin. It was inactive against 'Crill 6' (polyoxyethylene derivative of sorbitan laurate) by either method and the substrates tributyrin and olive oil were found not to be interchangeable in the two methods of assay.

Possible activation. Samples (0·5 ml.) of a purified lipase preparation (0·25 mg./ml. dry matter, 0·38 lipase units/ml.) were used to examine the influence of various substances upon the initial reaction velocity, with tributyrin as substrate. The addition of 0·5 ml. of 0·01 M-$CaCl_2$, $MgSO_4$ and $MnSO_4$ or 0·5 ml. of 0·5% (w/v) 'Crill 6', egg albumin, sodium oleate and sodium glycocholate caused neither activation nor inhibition.

DISCUSSION

The difficulties of differentiating lipase and esterase activities and the choice of substrate for measuring lipolytic activity have recently been discussed by Desnuelle (1951). It is generally considered that tributyrin hydrolysis is effected by a true lipase and, in the case of milk, the 'tributyrinase' and lipase activities have definitely been correlated (Dunkley & Smith, 1951). Evidence is presented in this paper that the same enzyme is responsible for the hydrolysis of olive oil and tributyrin since the ratio of the activities of the enzyme, at various stages of purification, against these two substrates, measured by two different methods, remains the same. These two substrates are not, however, interchangeable in the two methods of assay described. Hutchinson & Martin (unpublished data) have shown that oat lipase activity against olive oil in a dough decreases with increased water (above 40%) until it is practically zero in a system which is predominantly aqueous: this would explain the non-hydrolysis of olive oil in the manometric system. Balls & Tucker (1938) have reported pancreatic lipase activity against both olive oil and tributyrin at low temperatures (−15 to −38°) where the system (which was predominantly aqueous) was solid.

This suggests that oat lipase differs from pancreatic lipase and a survey of the reported properties of lipases from various sources indicates that they may all differ from each other, judged by the criteria of solubility, specificity and kinetic properties. Thus wheat-germ lipase has been assayed against α-monobutyrin and 'Tween 20' (polyoxethylene sorbitan laurate) (Singer & Hofstee, 1948) and the lipase of *Fusarium lini* against triacetin (Fiore & Nord, 1950). Oat lipase has been shown to be completely inactive against the two former substrates and to hydrolyse triacetin at only one-third the rate it hydrolyses tributyrin although triacetin is water-soluble.

The rate of hydrolysis of tributyrin by the purified oat lipase is unaffected by the presence of dispersing agents such as 'Crill 6', egg albumin, glycocholate, sodium oleate, calcium, magnesium or manganese ions. In this respect it differs from the pancreatic lipase studied by Willstätter & Memmen (1924).

Differences between lipase preparations from different sources are reflected in their solubility relations. The only other lipase preparation reported to be water-insoluble is that from castor-bean but this is soluble in ether (Takamiya, 1935). The oat-lipase preparation described in this paper is insoluble in a wide range of solvents.

The kinetic properties of oat lipase resemble, more closely, those of the wheat-germ lipase described by Singer & Hofstee (1948) than any of the other lipases, but differ from the latter in solubility behaviour and in specificity towards different substrates.

Takamiya (1936) has demonstrated that *Ricinus* lipase requires an activator and Kraut & Pantschenko-Jurewicz (1934) and Schønheyder & Volquartz (1946) have shown that pancreatic lipase can be resolved into apoenzyme and coenzyme moieties. The inactivation of the oat enzyme by precipitation with ammonium sulphate and isoelectric precipitation at pH 5·5 might possibly be due to a dissociation of a coenzyme factor. However, experiments on the recombination of fractions provided no evidence for this suggestion. The increase observed in total lipase

activity in the fractions obtained by scrubbing the groats compared with that found in the original whole groats is thought to be due to differences in physical factors.

The purified preparation has a nitrogen content of 6·7 %. If the enzyme is an unconjugated protein the preparation is therefore far from pure. On the other hand, it is possible that the preparation does not contain as much inert material as the low nitrogen content would suggest but that, as the physical properties appear to indicate, oat lipase has an unusual constitution.

A high production of diglyceride has been observed with certain preparations of pancreatic lipase (Desnuelle, Naudet & Rouzier, 1948), and Frazer (see Desnuelle, 1951) has suggested that more than one enzyme may be involved in the complete hydrolysis of a triglyceride. Recently Mattson, Benedict, Martin & Beck (1952) have put forward evidence for selective hydrolysis by rat pancreatic lipase resulting in the formation of $\alpha\beta$-diglycerides and β-monoglycerides. In this paper it has been shown that the purified oat lipase will specifically split off one butyric acid radical from tributyrin and this fact would appear to give weight to the *in vivo* studies of Mattson *et al.* (1952) and the suggestion of Frazer.

SUMMARY

1. The lipase causing lipolysis in oatmeals has been shown to be a true cereal lipase, not a product of the subepidermal fungal contaminants.

2. This lipase has been purified some 2000-fold on a dry-matter basis compared with the activity in the original oatmeal. The purified preparation contained 6·7 % nitrogen and 0·5 % phosphorus.

3. The purified preparation has an optimum pH of 7·4, optimum temperature 37–38°, and a Michaelis-Menten constant (K_m) of 0·006 M when tributyrin is the substrate.

4. The purified lipase splits off one butyric acid radical only from tributyrin and does not hydrolyse the various mono- and di-butyrins at pH 7·4 and 37°.

5. The oat lipase will not attack the polyoxyethylene derivative of sorbitan laurate nor is it 'activated' by the compensatory activators required, according to Willstätter, by pancreatic lipase.

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Oxidative Phosphorylation Coupled with the Oxidation of α-Ketoglutarate by Heart-muscle Sarcosomes

1. KINETICS OF THE OXIDATIVE PHOSPHORYLATION REACTION AND ADENINE NUCLEOTIDE SPECIFICITY

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Ochoa (1944) showed that inorganic phosphate was esterified when α-ketoglutarate was oxidized to succinate by particulate preparations obtained from heart muscle. These particles have been identified with the sarcosomes, the granules in muscle which correspond to mitochondria in other tissues (Cleland & Slater, 1953*a*, *b*; see also Harman & Feigelson, 1952).

A recent paper (Slater, 1953*b*) described a procedure for measuring the yield of oxidative phosphorylation, in which adenosine diphosphate (ADP), glucose and yeast hexokinase were added to the reaction mixture, and the hexose monophosphate (HMP) formed was determined in the deproteinized mixture by a sensitive enzymic method (Slater, 1953*a*). The reactions occurring may be written as follows, in which ATP = adenosine triphosphate:

$$\alpha\text{-ketoglutarate} + \tfrac{1}{2}O_2 + x\text{ADP} + x\text{H}_3\text{PO}_4 \rightarrow \text{succinate} + \text{CO}_2 + x\text{ATP} + x\text{H}_2\text{O}, \quad (1)$$

$$x\text{ATP} + x\text{glucose} \rightarrow x\text{HMP} + x\text{ADP}. \quad (2)$$

Overall reaction

$$\alpha\text{-ketoglutarate} + \tfrac{1}{2}O_2 + x\text{glucose} + x\text{H}_3\text{PO}_4 \rightarrow \text{succinate} + \text{CO}_2 + x\text{HMP} + x\text{H}_2\text{O}. \quad (3)$$

Equation (1) summarizes the reactions of oxidative phosphorylation coupled with the oxidation of α-ketoglutarate to succinate; these are brought about by the sarcosomes, while reaction (2) is catalysed by the yeast hexokinase added. In this procedure, ADP acts as a catalyst. A slow side reaction, catalysed by myokinase and hexokinase and leading to the phosphorylation of glucose by ADP (equation 4), is allowed for in a control measurement (the myokinase control) in which no oxidation occurs (AMP = adenosine monophosphate):

$$\text{ADP} + \text{glucose} \rightarrow \text{AMP} + \text{HMP}. \quad (4)$$

The method of determining HMP depends upon its enzymic conversion in the presence of excess ATP to dihydroxyacetone phosphate, which then reacts with reduced diphosphopyridine nucleotide (reduced DPN), a reaction catalysed by glycerol phosphate dehydrogenase. The amount of reduced DPN is determined spectrophotometrically. The use of this sensitive method of measuring the HMP formed enabled the kinetics of the early stages of the overall reaction (3) to be followed. For the study of the kinetics of the oxidative phosphorylation reaction, it is necessary either that reaction (2) should be very rapid in comparison with reaction (1) or that reaction (1) should be studied separately. Ways of doing this and the results obtained are considered here.

This paper also deals with the specificity of ADP as the phosphate acceptor in reaction (1). Ochoa (1944) found that AMP as well as ADP and ATP could catalyse the uptake of inorganic phosphate while Lehninger & Smith (1949) found that AMP and ADP were equally effective. The only adenine nucleotide used in the detailed study of oxidative phosphorylation by Cross, Taggart, Covo & Green (1949) was AMP.

As already briefly mentioned in connexion with a study of the phosphorylation coupled with the reduction of cytochrome *c* by α-ketoglutarate (Slater, 1950*a*), it was found that AMP was phosphorylated by heart preparations much more slowly than ADP, showing that ADP is the preferred primary phosphate acceptor. This has been confirmed by Barkulis & Lehninger (1951) and Kielley & Kielley (1951) with liver preparations and by Lindberg & Ernster (1952) with a heart preparation.

Barkulis & Lehninger (1951) found that fluoride inhibited the phosphorylation of AMP but not of ADP, and suggested that ADP was the primary phosphate acceptor and that the phosphorylation of AMP required the participation of the fluoride-sensitive myokinase. Their experiments did not, however, exclude the possibility that AMP might still be directly phosphorylated at an appreciable rate, a reaction which could be important in initiating the uptake of inorganic phosphate. Lindberg & Ernster (1952) believe that a direct

phosphorylation of AMP does in fact occur, and that this reaction is essential for oxidative phosphorylation. The finding of Lipmann, Jones, Black & Flynn (1952) that the 'activation' of acetate by ATP involves the reversible reaction (5)

$$\text{coenzyme A pyrophosphate} + \text{AMP} \rightleftharpoons \text{coenzyme A} + \text{ATP} \quad (5)$$

suggests the possibility of a similar reaction in the phosphorylation coupled with the oxidation of α-ketoglutarate, which also requires coenzyme A (Kaufman, 1951).

The analytical methods already mentioned (Slater, 1953*a*) permitted the direct testing of the adenine nucleotide specificity of the oxidative phosphorylation reaction. A preliminary account of this has already been published (Slater & Holton, 1953).

METHODS

Sarcosomal preparations. Two main types of preparation were used in this study.

(1) Except where stated otherwise, the preparations were made from heart immediately after killing the animal, using the isolation media and procedures described by Cleland & Slater (1953*a*). Different types of preparation within this main type are designated according to the isolation medium and the animal used.

(2) Preparations made from pig heart obtained from the slaughter house. The heart was placed in ice soon after removal from the animal and the preparation commenced about 1 hr. later.

Measurement of activity of α-ketoglutaric oxidase system and yield of oxidative phosphorylation. Except where stated otherwise, the 'isotonic reaction mixture' containing fluoride described by Slater & Cleland (1953*a*) was used. Where O_2 uptake was measured, the cold sarcosome preparation (usually 0·1–0·15 ml. in a final vol. of 1 ml.) was added at zero time to the main compartment of a 7 ml. Warburg flask which already contained all the other components of the reaction mixture at room temperature. KOH (10%, w/v) and a filter-paper roll were used to absorb CO_2. The flask was immediately attached to the manometer and placed in a bath at 25°. Manometer readings were started 5–10 min. later and the O_2 uptake during the equilibration period determined by extrapolation. The validity of this procedure will be examined below. At the required time, the flasks were removed from the manometer and the reaction immediately stopped with trichloroacetic acid (TCA). The subsequent procedure and the methods of analysis of HMP and energy-rich phosphate (~P) have already been described (Slater, 1953*a*, *b*). A zero-time sample was also analysed and the values deducted from those obtained from the experimental samples to give ΔHMP and Δ~P.

Experiments of short duration were carried out in open Erlenmeyer flasks which could be shaken in the manometric bath. By using blow-out pipettes, accurate timing of the additions of the sarcosome preparation and TCA could be made.

Activity of α-ketoglutaric-cytochrome c reductase system. The isotonic reaction mixture was used with the addition of KCN (0·016 M). The α-ketoglutarate concentration was 0·003 M and the concentration of sarcosomes, 0·1 mg. protein/ml. The cyanide was added to the other components of the reaction mixture in a 1 cm. Beckman cell just before the sarcosomes which started the reaction. The total volume was 3 ml. The optical density at 550 mμ. was followed as a function of time for 3–4 min., using as reference cell a similar reaction mixture including cytochrome *c* and sarcosomes, but lacking cyanide and α-ketoglutarate. The sarcosomes were added to the reference cell a few minutes before the start of the reaction to allow complete oxidation of the ferrocytochrome *c* in the cytochrome *c* preparation. The temperature of the reaction mixture was determined (all were between 15° and 17°) and the measured rate corrected to 15°, assuming a temperature coefficient for the 10° range of 2. One unit of activity is defined as a rate of increase of optical density of 0·01/min., calculated from the initial rate.

Determination of α-ketoglutarate. In many of the experiments described in the present paper, the disappearance of α-ketoglutarate ($-\Delta\alpha$Kg) was also determined. An enzymic method, depending upon reaction (6),

$$\alpha\text{-ketoglutarate} + NH_4^+ + \text{reduced DPN} \rightarrow \text{glutamate} + \text{DPN}^+, \quad (6)$$

catalysed by glutamic dehydrogenase was used (cf. Kornberg & Pricer, 1951). The amount of reduced DPN reacting with the α-ketoglutarate was estimated spectrophotometrically at 340 mμ., using the same procedure as that described for the estimation of hexose diphosphate (HDP) (Slater, 1953*a*). The Beckman cells contained 0·05 M-phosphate, pH 7·4, and 0·1 M-NH_4Cl, in addition to the reduced DPN and the sample to be analysed (containing about 0·1–0·15 μmoles α-ketoglutarate). This method is very reproducible, provided care is taken with the dilution of the solution to be analysed. This is necessary, since a small error in an individual analysis of a test solution or the zero-time control causes a much larger relative error in the value of $-\Delta\alpha$Kg.

Different preparations of glutamic dehydrogenase have been used for these measurements. If the reduced DPN preparation contains ethanol (e.g. if it is prepared by reduction with ethanol and alcohol dehydrogenase), the glutamic dehydrogenase should be purified to a stage where there is little residual alcohol dehydrogenase. Otherwise, reduction of the DPN will occur after reaction (6) has gone to completion. A satisfactory preparation from ox-liver acetone powder was obtained by following the procedure of Kornberg & Pricer (1951) up to the second fractionation with $(NH_4)_2SO_4$. The precipitate obtained was dissolved in a small volume of water and stored at −15°. This preparation retained sufficient activity for several weeks. The amount of enzyme required depends upon the amount of trichloroacetate in the sample, since trichloroacetate is a strong inhibitor of reaction (6). The reaction was usually complete within 5 min. of the addition of the enzyme and the reading remained stationary for some time. No extrapolation of the type described by Slater (1953*a*) is usually necessary for this measurement. The blank obtained in the absence of α-ketoglutarate is small.

Oxaloacetate and pyruvate interfere with this measurement owing to the presence of malic and lactic dehydrogenases, respectively. However, neither of these substances is present in the solution obtained after the oxidation of α-ketoglutarate by heart-muscle sarcosomes (Slater, 1953*b*).

HDP, HMP and ~P do not interfere. The recovery of even small amounts of α-ketoglutarate in zero-time controls was excellent (Table 1).

Table 1. *Recovery of α-ketoglutarate added to reaction mixtures*

(Known amounts of α-ketoglutarate were added to the zero-time control, containing sarcosomes and the complete reaction mixture.)

α-Ketoglutarate		
Added (μmoles)	Found (μmoles)	Recovery (%)
4·90	4·80	98
7·39	7·42	100
0·89	0·84	95
0·88	0·93	105
0·93	0·93	100
1·30	1·29	99

Myokinase. This was a preparation from rabbit muscle made by the method of Colowick & Kalckar (1943). It was kindly supplied by Dr G. Cantoni.

α-Ketoglutaric acid. Since commercial samples of α-ketoglutaric acid contain up to 10% succinic acid, they were purified by dissolving in hot acetone and crystallizing by the addition of benzene, followed by solution in hot acetic acid and crystallization by the addition of benzene. These purified preparations contained about 1% succinate, as measured manometrically with the Keilin & Hartree (1947) heart-muscle preparation. In most of the experiments described in this paper, a sample of α-ketoglutaric acid containing no measurable amount of succinate was used. This sample was kindly supplied by Dr L. Levenbook.

Other materials were as described in previous papers (Slater, 1953*a*, *b*). The unit of hexokinase is that of Berger, Slein, Colowick & Cori (1946).

RESULTS

Measurement of rate of oxidation

Owing to technical difficulties, it is not possible to measure with sufficient accuracy the rate of oxygen uptake in the first few minutes of a manometric experiment. Errors can become particularly serious when the oxygen uptakes are low and carbon dioxide is produced by the reaction, as is the case during the oxidation of α-ketoglutarate to succinate (R.Q. = 2). For the experiments described in this paper, it was found desirable to use differential manometers, and care was taken to provide a sufficient surface of filter paper, wet with potassium hydroxide, for the efficient absorption of carbon dioxide. Even under these circumstances, we have found that the manometric readings cannot be completely relied upon until about 7–10 min. after placing the flasks in the bath. We have, therefore, adopted the practice of extrapolating the readings to zero time in order to obtain the total oxygen uptake.

Fig. 1 describes two manometric records, curve 1 with a low concentration (0·12 mg. protein/ml.) of cat-heart sarcosomes, and curve 2 with the more usual concentration of rat-heart sarcosomes (0·60 mg. protein/ml.). The first point on each curve represents the first manometer reading. The oxygen uptake between zero time and the time of this reading was obtained by extrapolation and the value obtained added to all readings. Curve 1 illustrates the precision with which even very small oxygen uptakes can be measured with differential

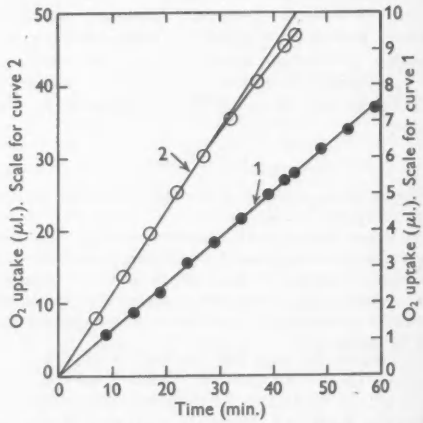


Fig. 1. Course of O_2 uptake in oxidation of α-ketoglutarate by heart sarcosomes in isotonic reaction mixture. Curve 1, cat-heart sarcosomes, 0·12 mg. protein/ml. Curve 2, rat-heart sarcosomes, 0·60 mg. protein/ml. Note different scales of the ordinates. See text for description of procedure.

manometers and small flasks (constant of manometer = 0·47). The cat-heart sarcosomes oxidized α-ketoglutarate at a uniform rate for at least 60 min., while the rat-heart sarcosomes showed a slight decline after about 30 min. These steady rates are obtained only if the inactivation of the α-ketoglutaric oxidase system is prevented by the addition of ethylenediaminetetraacetic acid (Versene) (Slater & Cleland, 1952).

The extrapolation procedure is valid only if the rate of oxidation during the equilibration period is the same as that measured later. In particular, it might be imagined from the frequent behaviour of the manometric readings in the first few minutes (not shown in Fig. 1) that there is an initial lag period in the oxidation. The following evidence shows that there is no lag.

(1) Replicate flasks corresponding to curve 2, Fig. 1, were removed and analysed after 15, 30 and 45 min., with the results shown in Table 2. It can be seen that the oxygen uptakes, calculated assuming

the validity of the extrapolation, agree closely with the amounts of α-ketoglutarate disappearing. Figs. 2 and 4 show the disappearance of α-ketoglutarate at a uniform rate in a short-duration experiment.

Table 2. *Relationship between oxygen uptake, α-ketoglutarate disappearance and ΔHMP at different times*

(Replicate flasks, identical with that whose O_2 uptake is shown in curve 2, Fig. 1, were removed at different times and treated with trichloroacetic acid.)

Time (min.)	O_2 uptake ($-\Delta O$)* (μg. atoms)	$-\Delta\alpha$Kg (μmoles)	ΔHMP† (μmoles)	P:O
15	1·57	1·58	4·31	2·75
30	3·13	3·19	8·46	2·71
45	4·31	4·35	11·78	2·73

* O_2 uptake in first 7 min. obtained by extrapolation.
† Corrected for myokinase control (Slater, 1953*b*).

(2) When cytochrome *c* was used as hydrogen acceptor instead of oxygen (in the presence of cyanide or in the absence of oxygen), there was usually no lag in the rate of reduction of cytochrome *c* (e.g. see the fig. of Slater, 1950*a*).

Kinetics of the oxidative phosphorylation

In earlier experiments (Slater, 1950*a*), it was found that phosphorylation, measured by the formation of HMP, did not begin immediately on adding the sarcosomes to the reaction mixture. An example is shown in curve 1, Fig. 2. Since there is no lag in the rate of oxidation, this lag of phosphorylation behind the hydrogen transfer must be due to the accumulation of some substance which is formed intermediate between the hydrogen-transfer reaction and the HMP. As already discussed (Slater, 1950*a*), this intermediate could be either one formed in the oxidative phosphorylation reaction (1) (succinyl phosphate seemed a possibility at that time) or ATP. There must, in fact, be some accumulation of ATP; its magnitude will depend upon the relative rates of reactions (1) and (2). The greater the rate of the hexokinase reaction relative to the rate of synthesis of ATP by reaction (1), the less will be the steady-state concentration of ATP.

The possibility that in addition to ATP some other intermediate product might be responsible for the lag was tested by omitting the hexokinase and measuring the rate of increase of ~P. This measures directly the rate of phosphorylation without the necessity of correcting for the myokinase activity since the myokinase reaction does not alter the total ~P content. In order to avoid the technical difficulties of carrying out experiments for very short times, a low concentration of sarcosomes (0·12 mg. protein/ml.) was used. The results of this experiment, given in Fig. 3, curve 1, show that there is no measurable lag in ~P formation. There is, therefore, no evidence for any accumulation of an intermediate in the oxidative phosphorylation reaction itself.

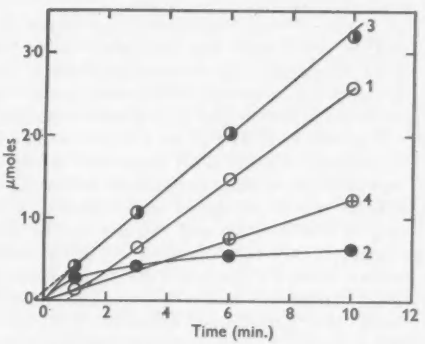


Fig. 2. Kinetics of early stages of oxidative phosphorylation using small amount of hexokinase (13 units/mg. sarcosomal protein). Isotonic sucrose-ethylenediaminetetraacetate rat-heart sarcosomes, 0·72 mg. protein/ml.; initial ~P, 0·61 μmole. Curve 1, ΔHMP; curve 2, Δ~P; curve 3, ΔHMP+Δ~P; curve 4, $-\Delta\alpha$-ketoglutarate.

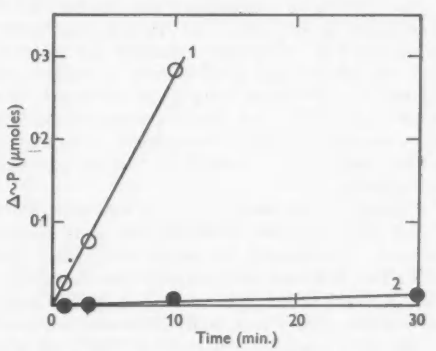


Fig. 3. Rate of phosphorylation of ADP (curve 1) and of AMP (curve 2), in the absence of hexokinase + glucose. Isotonic reaction mixture, except for omission of hexokinase and glucose and of AMP from curve 1 and ADP from curve 2. Isotonic sucrose-ethylenediaminetetraacetate cat-heart sarcosomes, 0·12 mg. protein/ml.

The P:O ratio corresponding to Fig. 3, curve 1, is 2·4, compared with 3·0 obtained in the presence of added hexokinase and glucose. The slightly lower phosphorylation yield obtained without hexokinase is certainly due to the action of ATP-ase which

would cause hydrolysis of ATP, the end product of the reaction in the absence of hexokinase. The ATP will react with glucose instead of water and accumulate as HMP when sufficient hexokinase is present.

The conclusion that the phosphorylation kinetics are perfectly linear was confirmed by measuring the course of the synthesis of HMP + ~P, in the presence of hexokinase and glucose.

Fig. 2 describes an experiment in which the amount of hexokinase was purposely made low (13 units hexokinase/mg. sarcosomal protein). The separate myokinase control test (Slater, 1953*b*) was not carried out; instead, each solution was analysed for ~P as well as HMP. If no ATP accumulated, ~P would fall linearly with time as a result of the combined myokinase-hexokinase reaction (4), while HMP would increase linearly with time as a result of reactions (3) and (4). In fact, in this experiment, there was a marked lag in the HMP formation, while ~P instead of decreasing actually increased because the rate of accumulation of ATP exceeded the hexokinase reaction. The curve (ΔHMP + Δ~P), however, showed no lag. It is probable that the rate of phosphorylation was, in fact, somewhat greater during the first minute. This is understandable, since in the presence of only a small amount of hexokinase there will be hydrolysis of ATP byATP-ase. While the ATP concentration is increasing, this loss of esterified phosphate will be less than when it reaches a steady state.

Fig. 4 shows an experiment with the amount of hexokinase usually employed (99 units/mg. sarcosomal protein). Although a distinct lag phase in both the ΔHMP and Δ~P curves is evident, the (ΔHMP + Δ~P) is perfectly linear for 4 min. The subsequent fall in the rate of phosphorylation in this experiment is due to exhaustion of substrate, a low concentration (0·0012 M) of which was used (see curve 4).

By using a low concentration of sarcosomes and a high concentration of hexokinase, it is possible virtually to eliminate the initial accumulation of ATP. This is shown in the experiment described in Fig. 5, in which 510 units of hexokinase/mg. sarcosomal protein were used. Two sets of measurements were made (i) with added ADP, (ii) with added AMP and ADP. The latter is the usual procedure in the method of measuring the yield of oxidative phosphorylation previously described (Slater, 1953*b*), the AMP being added as an inhibitor of myokinase. (The addition of AMP has no effect on the P:O ratio.) With the low concentration of cat-heart sarcosomes used, there was no measurable decline of the ~P after 60 min. in the presence of AMP. In the absence of AMP, ~P declined at a slow but linear rate, due to reaction (4).

The finding that the accumulation of ~P is virtually eliminated by using a sufficient concentration of hexokinase shows that the ~P compound accumulating is in fact ATP and not a compound of the type of succinyl phosphate which could conceivably be measured as ~P by the analytical method used.

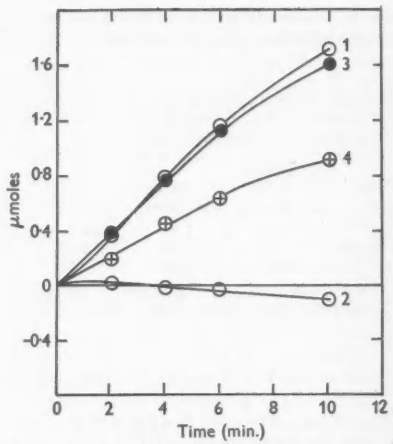


Fig. 4. Kinetics of early stages of oxidative phosphorylation, using normal amount of hexokinase (99 units/mg. sarcosomal protein). Saline-ethylenediaminetetraacetate cat-heart sarcosomes, 0·64 mg. protein/ml. Curve 1, ΔHMP; curve 2, Δ~P; curve 3, ΔHMP + Δ~P; curve 4, $-\Delta\alpha$-ketoglutarate.

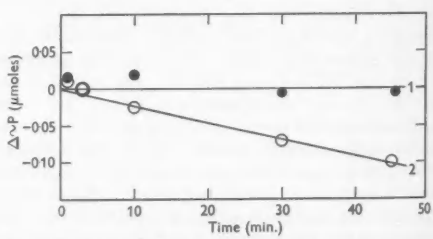


Fig. 5. Kinetics of oxidative phosphorylation, using large excess of hexokinase (510 units/mg. sarcosomal protein). Curve 1, isotonic reaction mixture; curve 2, same reaction mixture, except no AMP. Same preparation and same concentration of sarcosomes as in Fig. 3.

The effect of varying the amount of hexokinase on the steady-state level of ~P was further studied in the experiment described in Fig. 6. In this experiment, the oxygen uptake substantially exceeded the α-ketoglutarate disappearance, indicating considerable oxidation past the succinate stage. Both oxygen uptake and α-ketoglutarate disappearance were inhibited by the larger amounts of hexokinase. The yield of oxidative phosphorylation has been expressed both as P:O and as P:αKg.

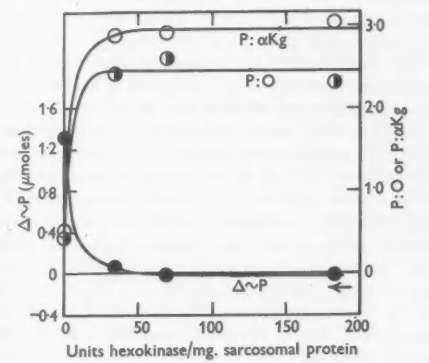
The reactions taking place under the conditions of the experiment may be described by

$$ADP + H_2PO_4 \rightarrow ATP + H_2O \text{ (\textit{oxidative phosphorylation})}, \quad (1)$$

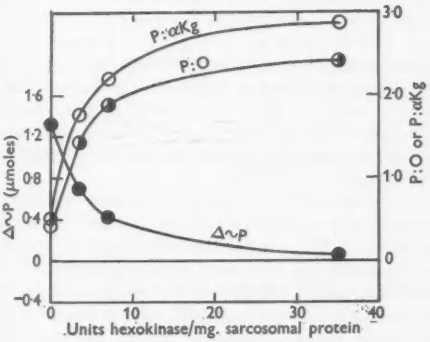
$$ATP + H_2O \rightarrow ADP + H_3PO_4 \text{ (\textit{ATP-ase})}, \quad (7)$$

$$ATP + AMP \rightleftharpoons 2ADP \text{ (\textit{myokinase})}, \quad (8)$$

$$ATP + \text{glucose} \rightarrow ADP + HMP \text{ (\textit{hexokinase})}. \quad (2)$$



(*a*)



(*b*)

Fig. 6. (*a*) Effect of amount of hexokinase on the measured yield of oxidative phosphorylation (P:O and P:αKg) and on Δ~P. Esterified P = ΔHMP + Δ~P. Saline-ethylenediaminetetraacetate rat-heart sarcosomes, 1·45 mg. protein/ml. Initial ADP, 0·6 μmole; AMP, 0·6 μmole. Hexokinase used had specific activity of approx. 750 units/mg. protein. Horizontal arrow shows Δ~P found in a separate 'myokinase control' (see text).

(*b*) Same experiment, showing values obtained with 0–35 units hexokinase/mg. sarcosomal protein.

In the absence of hexokinase, the steady state concentration of ~P will be governed by the relative rates of reactions (1), (7) and (8), including the back reaction of equation (8). The Δ~P found under these conditions (1·33 μmoles) is considerably greater than the ADP originally used (0·6 μmole) showing that some of the AMP (originally 0·6 μmole) has been phosphorylated via reaction (8). The total ~P found was 80% of the value which would be obtained if all the AMP and ADP had been converted to ATP.

Even small amounts of hexokinase (3·5–7 units/mg. sarcosomal protein) greatly decreased the ~P and increased the total amount of esterified P (ΔHMP + Δ~P). However, this amount of hexokinase was not sufficient to transfer all the ATP formed by reaction (1) to glucose, so that considerable amounts of ~P accumulated. Moreover, under these conditions, the total esterified P was smaller in amount than that found with larger quantities of hexokinase. This is because the ATP-ase competes with these small amounts of hexokinase and causes loss of esterified P. In this experiment, 35 units of hexokinase/mg. sarcosomal protein were sufficient to give optimum esterification of inorganic phosphate, the P:O and P:αKg remaining virtually constant when the amount of hexokinase was varied between 35 and 182 units/mg. sarcosomal protein. The Δ~P was very low under these conditions. The arrow on the lower right-hand corner of Fig. 6*a* shows the Δ~P found in a separate myokinase control, carried out in the presence of cyanide; this is due to the reaction shown in equation (4). The amount by which Δ~P exceeds this value (except when there was very little hexokinase) equals the amount of ATP present in the reaction mixture. With 70 or more units of hexokinase/mg. sarcosomal protein, this was only about 0·12 μmole ATP. It is noteworthy that the loss of esterified P, indicated by the point on the curves in Fig. 6 at which the P:O or P:αKg reached a maximum, ceased when the steady-state concentration of ATP had reached a very low value. This is exactly what would be expected from a consideration of equations (1), (7), (8) and (2).

The measurement of ΔHMP + Δ~P shown in Fig. 6 and in earlier experiments in this section obviates the need for the separate myokinase control, necessary in the procedure described by Slater (1953*b*) in which only ΔHMP is measured. It is also strictly more correct, since it allows for the ATP as well as the HMP. However, the procedure previously described is often more convenient, since it requires fewer analyses. The error is usually completely negligible, provided sufficient hexokinase is used, which is in any case essential to obtain the true value for the P:O ratio. For example, in the experiment shown in Fig. 6, with 70 units of hexokinase/mg. sarcosomal protein, the ATP concentration was only 1% of that of the HMP.

Adenine nucleotide specificity

Fig. 7 shows the oxygen uptake for the period 15–45 min. using a dilute sarcosomal preparation with the same concentration (about 0·0006 M) of different adenine nucleotides, both in the presence and absence of hexokinase. No attempt was made to extrapolate to zero time, since in the absence of hexokinase there was a decreasing rate of oxygen uptake. It is probable that by the time the first manometric reading was taken, nearly all the ADP would be phosphorylated to ATP in the absence of hexokinase.

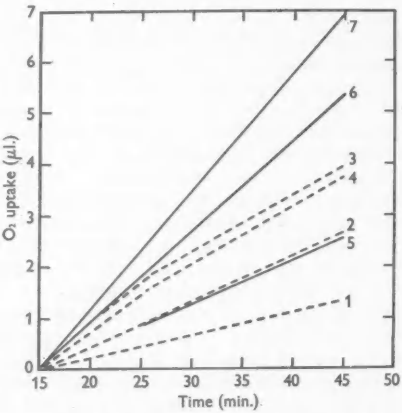


Fig. 7. Comparison of different adenine nucleotides. Isotonic sucrose-ethylenediaminetetraacetate cat-heart sarcosomes, 0·15 mg. protein/ml. Isotonic reaction mixture except for adenine nucleotide and hexokinase + glucose. Curves 1 and 5, no added nucleotide; curves 2 and 6, AMP (0·6 μmole); curves 3 and 7, ADP (0·53 μmole); curve 4, ATP (1·02 μmole ~ P). Without hexokinase (discontinuous lines); with 2660 units hexokinase/mg. sarcosomal protein + glucose (continuous lines). O_2 uptakes between 15 min. and 45 min. after adding sarcosomes.

The first point to note is that, either in the absence of adenine nucleotide or in the presence of AMP or ADP, hexokinase stimulated the oxygen uptake, especially after 25 min. This is in agreement with several recent authors who used ATP as the adenine nucleotide (e.g. Rabinovitz, Stulberg & Boyer, 1951; Lardy & Wellman, 1952). Secondly, there was appreciable oxidation in the absence of added nucleotide, especially in the presence of hexokinase. Both AMP and ADP stimulated the oxygen uptake, but the latter was the more effective, especially in the absence of hexokinase.

The HMP and ~P contents at the end of the experiment shown in Fig. 7 are given in Table 3. The following points are worthy of comment.

(1) The ADP was completely phosphorylated. From data not shown in Table 3, it can be calculated that the ratio of ~P to total adenosine in no. 3 of Table 3 was 2·08 (cf. theoretical value for ATP, 2·00). The slight increase of ~P in no. 4 is due to the presence of some ADP in the sample of ATP used.

(2) In contrast, there was very little phosphorylation of AMP, only 0·035 μmole ~ P. Since ADP is rapidly and completely phosphorylated, it follows that in the absence of hexokinase this ~P must all be ATP; thus only 0·017 μmole AMP was phosphorylated, which is only 0·6 % of the rate of phosphorylation of which the preparation was capable (no. 7, Table 3).

(3) The sarcosomes, even in the absence of any added adenine nucleotide, were able to synthesize appreciable amounts of HMP when hexokinase and glucose were added. Since ATP is the specific phosphate donor for hexokinase, this must be due to endogenous adenine nucleotide in the sarcosomal preparation.

(4) Added AMP was almost as effective as ADP in catalysing the oxidation and the P : O ratio was also quite high. However, only 0·01 μmole of ~P was synthesized, i.e. the rate of phosphorylation of AMP was only 0·7 % that of the phosphorylation of hexose under these conditions. (This amount of ~P is close to the limit of sensitivity of the analytical

Table 3. *Effects of different adenine nucleotides on rate of oxidation and on phosphorylation*

(Isotonic sucrose-ethylenediaminetetraacetate cat-heart sarcosomes, 0·15 mg. protein/ml. Analyses of experiment described in Fig. 7. Reaction period, 45 min.)

No.	Nucleotide	Hexokinase + glucose	$-\Delta O$ (μg. atoms)	ΔHMP (μmoles)	$\Delta \sim P$ (μmoles)	$\frac{(\Delta HMP + \Delta \sim P)}{-\Delta O}$
1	.	−	0·17	.	.	.
2	AMP	−	0·35	.	0·035	.
3	ADP	−	0·55*	.	0·72	.
4	ATP	−	0·51*	.	0·07	.
5	.	+	0·35	0·18	.	0·50
6	AMP	+	0·72	1·51	0·01†	2·10
7	ADP	+	0·93	2·84	. −0·14	2·89

* Extrapolation to zero time uncertain (see Fig. 7).
† Equivalent to a concentration of 10^{-5} M-ADP.

Table 4. *Effect of concentration of added ADP on activity of α-ketoglutaric oxidase system and on* P:O *ratio*

(Isotonic sucrose-ethylenediaminetetraacetate rat-heart sarcosomes, 1·20 mg. sarcosomal protein/ml.; 63 units hexokinase/mg. sarcosomal protein. AMP was added in concentration equal to that of added ADP.)

[ADP] (M × 10^4) Initial	[ADP] (M × 10^4) Final*	O_2 uptake (μg. atoms)	ΔHMP (μmoles)	P:O
0	0	4·37	5·40	1·24
0·98	0·28	5·75	15·6	2·72
3·0	—	5·59	15·2	2·72
6·1	4·1	5·77	15·6	2·70
12·0	—	5·09	13·9	2·74

* Determined by value of ΔHMP in myokinase control.

method and the value is therefore only approximate.) This shows unequivocally that AMP cannot be acting directly as an intermediate in the phosphorylation of glucose. For if AMP were directly phosphorylated to ATP, according to a reaction analagous to reaction (5), we should have

$$\text{AMP} \rightarrow \text{ATP}, \qquad (9)$$

$$\text{ATP} + \text{glucose} \rightarrow \text{ADP} + \text{HMP}, \qquad (2)$$

$$sum \quad \text{AMP} + \text{glucose} \rightarrow \text{ADP} + \text{HMP},$$

and there would be an increase of ~P as well as of HMP.

A possible explanation of the stimulating effect of adding the AMP is the presence of traces of ~P in the AMP solution used, which according to analysis

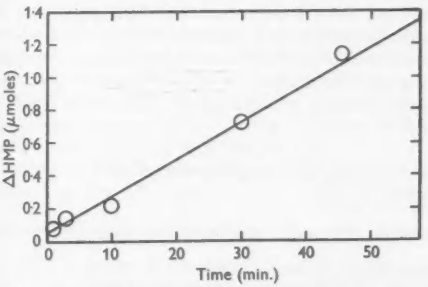


Fig. 8. Kinetics of oxidative phosphorylation with AMP as only added nucleotide. Same preparation and same conditions as in Fig. 5, except that AMP was the only added nucleotide.

of the stock solution of AMP would introduce 0·002 μmole ~P (2×10^{-6} M) into the reaction mixture. This explanation implies that a very low concentration of ADP is sufficient for optimum oxidation and phosphorylation, and has been confirmed by a different type of experiment summarized in Table 4, in which different concentrations of ADP were used. This experiment was carried out with a much higher concentration of rat-heart sarcosomes, so that the myokinase activity was appreciable, causing a considerable decline of the ADP concentration during the experiment. Both oxidation and phosphorylation were optimum when the concentration of ADP was $9{\cdot}8 \times 10^{-5}$ M at the beginning and $2{\cdot}8 \times 10^{-5}$ M at the end of the experiment. Table 3, no. 6, shows that 10^{-5} M-ADP was sufficient to give 77 % of the optimum rate of oxidation. This corresponds to a Michaelis constant of about 3×10^{-6} M-ADP (see also below).

Fig. 3 describes another experiment which directly compares the rate of phosphorylation of AMP with that of ADP, in the absence of hexokinase. The rate of increase of ~P with AMP was only about 1·5 % that with ADP, corresponding to a rate of phosphorylation of AMP of about 0·7 % that of ADP. In the presence of hexokinase (not shown in Fig. 3), no ~P could be detected (certainly less than 0·02 μmole) after 45 min., when AMP was the added nucleotide.

The kinetics of the synthesis of HMP with AMP as the only adenine nucleotide is shown in Fig. 8. Within experimental error, the rate is constant.

Fig. 9 describes a similar experiment with sarcosomes prepared from slaughter-house pig heart. These preparations differ in two important respects from the type used in the experiments described above. (1) Although they oxidize α-ketoglutarate rapidly, the P:O ratio is very low (usually less than 1), even under optimum conditions. These are the only preparations we have found with this characteristic. After a number of different types of treatment including incubation, it was the ability to oxidize α-ketoglutarate which was affected, not the P:O ratio (see Slater & Cleland, 1953*a*). (2) Pig-heart sarcosomes contain considerably more myokinase (Slater, 1953*b*).

The preparation of AMP used in this experiment (Fig. 9) contained some substance analysing as HMP. Over the first 8 min., there was actually a decline in the HMP content, due no doubt to the presence of a little phosphatase in pig-heart preparations (Slater, 1953*b*). Thereafter, the rate of phosphorylation steadily increased and was still increasing after 30 min., although the oxidation of α-ketoglutarate proceeded rapidly without any lag.

The maximum P:O ratio, calculated from the tangent of the curve in Fig. 9 at 30 min. was only 0·5.

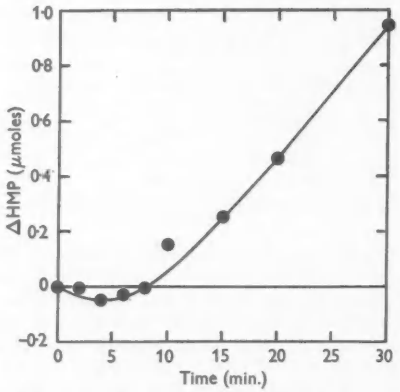


Fig. 9. Kinetics of oxidative phosphorylation, with AMP (0·001 M) as the only added nucleotide and sarcosomes prepared from slaughter-house pig heart (phosphate-saline preparation). Reaction mixture did not contain malonate or fluoride. 0·58 mg. sarcosomal protein/ml.

Fig. 10 describes an experiment with a similar preparation, in which the phosphorylation of AMP is compared with that of ADP. The course of the phosphorylation with AMP (curve 1) as acceptor was the same as that in Fig. 9, but with ADP (curve 2) there was only a very slight lag, of the same type as shown in Fig. 2. The addition of myokinase did not abolish the initial lag with AMP, but decreased the time required for phosphorylation to reach a maximum. The P:O ratio with ADP as acceptor was only 0·32 in this experiment.

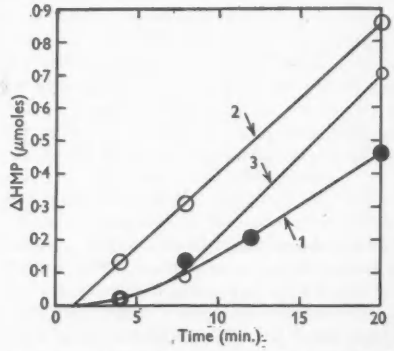


Fig. 10. Comparison of AMP and ADP as primary phosphate acceptors with sarcosomes prepared from slaughter-house pig heart (phosphate-saline preparation). Reaction mixture did not contain malonate or fluoride. 0·65 mg. sarcosomal protein/ml. Curve 1, AMP (0·001 M); curve 2, ADP (0·001 M); curve 3, AMP + rabbit-muscle myokinase.

Effect of concentration of sarcosomes

The rate of oxidation in the absence of added nucleotide was relatively much greater in the experiment described in Table 4 (77 % of the rate obtained with ADP) than in that described in Table 3 (38 %). The relative P:O ratios show a similar difference. These discrepancies are due to the different concentrations of sarcosomes used, the effect of which is more fully examined in Table 5

Table 5. *Effect of concentration of sarcosomes on rate of oxidation of α-ketoglutarate and on* P:O *ratio, in presence and in absence of ADP*

(Isotonic sucrose-ethylenediaminetetraacetate rat-heart sarcosomes; 146 units hexokinase/mg. sarcosomal protein; ADP, where added, 6×10^{-4} M.)

	Sarcosomal protein (mg./ml.)			
	0·12	0·31	0·62	1·25
O_2 uptake (μg. atoms)				
Without ADP (*a*)	0·26	0·64	1·68	4·15
With ADP (*b*)	0·70*	1·76	3·56	6·26†
$100 \times a/b$	37	36	47	63
ΔHMP (μmoles)				
Without ADP (*c*)	0	0·19	1·24	4·47
With ADP (*d*)	1·82	4·36	8·46	15·8†
$100 \times c/d$	0	4	15	28
P:O				
Without ADP (c/a)	0	0·33	0·74	1·08
With ADP (d/b)	2·59	2·48	2·38	2·52

* O_2 uptake not accurately measured; this is interpolated value.

† This was the only concentration which showed some decline in the rate of O_2 uptake towards the end of the experiment, the final O_2 uptake being 7·5 % less than that which would have been obtained had the initial rate been maintained. For comparison with other sets of values, the experimental values have been multiplied by the factor 100/92·5.

and Fig. 11. In the presence of ADP, both the oxygen uptake and the ΔHMP were approximately proportional to the concentration of sarcosomes. (The departure from linearity at the highest concentration is probably due to inhibitory substances in the hexokinase preparation, which was added in amounts proportional to the concentration of

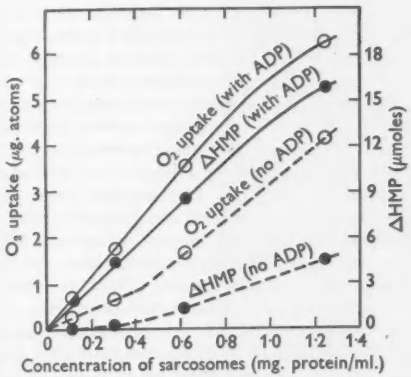


Fig. 11. Effect of concentration of sarcosomes on rate of oxidation of α-ketoglutarate and on ΔHMP, in presence and in absence of ADP. For experimental conditions, see Table 5.

sarcosomes.) The P:O ratio was, therefore, independent of the concentration of sarcosomes. In the absence of ADP, however, the oxygen uptake and more especially the ΔHMP were relatively much smaller at low concentrations of sarcosomes and the P:O ratio increased with increasing concentration of sarcosomes. This experiment shows that the endogenous adenine nucleotide of the sarcosomes is dissociated from the sarcosomes at high dilution.

Energy-rich P *content of sarcosomes*

Table 6 shows the results of three analyses of perchloric acid filtrates of freshly prepared sarcosomes. Considerable amounts of ~P (mean 0·02 μmole/mg. sarcosomal protein), easily measurable by the enzymic method, were detected. This value is very close to the acid-labile P content of liver mitochondria, namely 0·023 μmole/mg. protein (Kielley & Kielley, 1951). In analysis no. 3, the most accurate of the three, the ratio of ~P to total adenosine is 0·51. Since, as is discussed below, it is most likely that the ~P compound is ADP, other adenosine compounds are also present, probably AMP and possibly some coenzymes. The absorption spectrum of the neutralized perchloric acid filtrate was perfectly symmetrical around 260 mμ., showing the absence of other compounds absorbing in this region.

Table 6. *Analysis of sarcosomes*

(1–1·3 ml. sarcosomes (containing 4·8–11·6 mg. protein) were deproteinized with perchloric acid (final concentration 7%, w/v) and centrifuged. A sample of the supernatant was neutralized with 4N-KOH and, after standing at 0°, the precipitated $KClO_4$ removed by filtration. Samples of the filtrate were analysed by the procedure described by Slater (1953*a*). Preparation 1, isotonic saline-ethylenediaminetetraacetate rat-heart sarcosomes; preparation 2, isotonic sucrose-ethylenediaminetetraacetate cat-heart sarcosomes; preparation 3, isotonic sucrose-ethylenediaminetetraacetate rat-heart sarcosomes.)

	Preparation		
	1	2	3
Analyses (μmoles/mg. sarcosomal protein)			
Total adenosine	0·031	0·048	0·034
~P	0·02	0·027	0·018
HMP	—	0·026	—
HDP	—	—	0·002

Michaelis constant of ADP

The Michaelis constant (K_m) of the endogenous ADP may be calculated from the data of Table 5 and the ~P content of the sarcosomes, by using the expression $K_m = c(V/v - 1)$ where c is the concentration of ~P (mg. sarcosomal protein × 0·018), V (*b* or *d* in Table 5) is the velocity when the system is saturated with ADP and v (*a* or *c* in Table 5) is the velocity without ADP. The values obtained from these calculations are 10^{-5}M for oxidation and approx. 5×10^{-5}M for phosphorylation. The Michaelis constant is defined as the concentration of ADP necessary for half maximal activity; no other physical meaning is implied. In fact, these values of K_m are calculated on the basis of initial concentrations, and it would be expected that the ADP concentration would decrease, owing to the action of myokinase and hexokinase, during the course of the experiment (35 min. at 25°).

In order to measure the affinity of the system for adenine nucleotide more accurately, it was necessary to use a shorter experimental period. In the experiment summarized in Fig. 12, the oxidation of α-ketoglutarate by cytochrome *c* was measured spectrophotometrically, the measurements requiring only about 3 min. at 15°. Even at the very high dilution of sarcosomes used in this test, the endogenous ADP was sufficient for 76% of the maximal activity. It was possible, however, to measure the effect of very small concentrations of added ADP and, by plotting 1/activity against 1/[ADP], according to the procedure of Lineweaver & Burk (1934), to determine if there was any appreciable difference in activity of added and endogenous ADP. If the affinities of the system for endogenous and added ADP differed appreciably, a break in the curves between no added ADP and the first

addition would appear. Since no such break is apparent in Fig. 12, it is concluded that added and endogenous ADP behave similarly. The Michaelis constant calculated from Fig. 12 (*b*) is 7×10^{-7} M, considerably lower than that calculated from the experiment of longer duration. This may be partly due to the fact that the rate of oxidation of α-ketoglutarate by cytochrome *c* in the concentration used

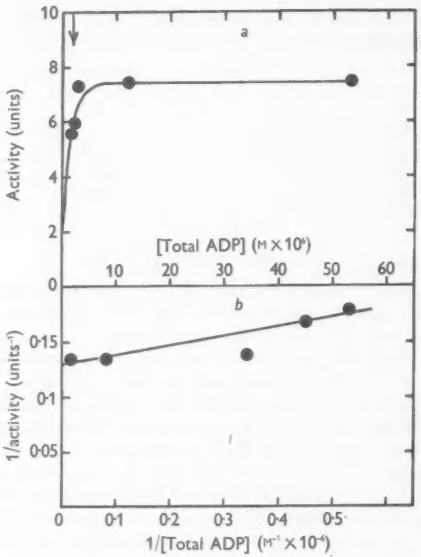


Fig. 12. Michaelis constant of ADP. The activity of the α-ketoglutarate-cytochrome *c* reductase system was determined as described in Methods. Isotonic sucrose-ethylenediaminetetraacetate rat-heart sarcosomes. The activities, both with and without ADP, declined on storage at 0° of the dilute sarcosome preparation during the course of the experiment and all activities were corrected to a common basis using appropriate decay curves. (*a*) The concentration of ADP provided by the sarcosomes is indicated by the vertical arrow. (*b*) Lineweaver-Burk (1934) plot.

in the spectrophotometric test is about 25–35 % of that by oxygen at the same temperature. The main reason, however, is probably the destruction of ADP in the longer experiment.

DISCUSSION

Adenine nucleotide specificity

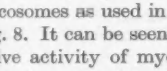
The above experiments show that the rate of phosphorylation of AMP is less than 1 % of that of ADP. Since such a slow rate of phosphorylation can easily be explained by a slight residual myokinase activity, it is reasonable to conclude that all the phosphorylation steps associated with the aerobic oxidation of α-ketoglutarate to succinate by heart-muscle sarcosomes are completely specific for ADP and do not react with AMP. The appreciable rate of phosphorylation of AMP which has been found by several previous authors cannot then be due directly to the oxidative phosphorylation, but must be entirely due to the myokinase reaction. The recent studies of Barkulis & Lehninger (1951) and of Kielley & Kielley (1951) clearly showed the participation of myokinase in the phosphorylation of AMP, but did not exclude the possibility of an additional relatively rapid direct phosphorylation of AMP. We can now see that the phosphorylation of AMP requires first the formation of ATP from ADP present either in the AMP preparation or in the sarcosomes or mitochondria and secondly sufficient myokinase to promote the phosphorylation of AMP. When hexokinase is also present, there must be a considerable myokinase activity to be able to compete with the hexokinase. This is the case with liver mitochondria which are rich in myokinase. Since the endogenous ADP is insufficient for optimal phosphorylation, the ADP formed from AMP by the myokinase reaction will stimulate the phosphorylation, thereby giving the appearance that AMP has entered into the oxidative phosphorylation reaction.

Provided there is sufficient myokinase, AMP will promote the uptake of inorganic P, either in the presence or the absence of hexokinase, without any detectable time lag. This is found with freshly prepared liver mitochondria (Kielley & Kielley, 1951). Ochoa's (1944) finding that AMP was effective with heart extracts is also understandable, since he used dialysed extracts which would be rich in myokinase. Cat or rat heart-muscle sarcosomes, however, contain only about 1 % of the myokinase activity of the extract (Cleland & Slater, 1953*a*). A stimulatory effect of AMP was found with pig-heart sarcosomes which are considerably richer in myokinase, but this was only obtained after a time lag. This lag was unduly prolonged in the experiments described in this paper, because the initial rate of phosphorylation of endogenous ADP was very low, due to the use of pig-heart muscle which was not completely fresh. It must be emphasized that this lag is of an entirely different order of magnitude and has quite a different explanation from that previously described (Slater, 1950*a*). As shown in Fig. 10, it disappears when ADP is the added nucleotide. It is thus also different from yet a third type of lag, reported by Kielley & Kielley (1951), who found that, after ageing, there was a considerable delay in reaching maximum phosphorylation rate with either AMP or ADP as acceptor. The reason for this third type of lag is

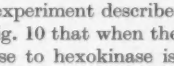
not known. Since Keilley & Keilley (1951) have measured only phosphorylation, not oxygen uptake, it is not known whether it is the rate of oxidation or the P:O ratio which is affected. Kielley & Kielley's lag may be similar to a lag of oxygen uptake found when heart-muscle sarcosomes are incubated at room temperature in the presence of ADP or ATP before addition of substrate (Slater & Cleland, 1953*b*).

The conclusion, that the oxidative phosphorylation reaction is completely specific for ADP and that the phosphorylation of AMP necessarily requires the participation of myokinase, differs from that drawn by Lindberg & Ernster (1952) from a study of the distribution of ^{32}P in various phosphorylated compounds after oxidative phosphorylation by rabbit-heart sarcosomes. These authors believe that AMP can be directly phosphorylated as well as ADP, although at only 5% of the rate of the latter. This conclusion is, however, entirely dependent upon the validity of the assumption that the hexokinase was so much in excess that the ATP would react entirely with glucose and not at all with AMP. Considering the much more concentrated preparations of sarcosomes used by these authors compared with our procedure, this seems to us to be very doubtful. The amount of hexokinase employed by Lindberg & Ernster (1952) is not stated, but it can be calculated that approximately 100 mg. of their hexokinase preparation would have been required (reaction vol. 10 ml.) to give the same ratio of hexokinase to sarcosomes as used in our experiment described in Fig. 8. It can be seen in Fig. 10 that when the relative activity of myokinase to hexokinase is increased, some ADP will be formed. We do not consider, therefore, that Lindberg & Ernster's experiments provide satisfactory evidence for the direct phosphorylation of AMP.

Krebs, Johnson, Eggleston & Hems (1951) have found by tracer experiments that the dephosphorylation and resynthesis of ATP in muscle suspensions occurred mainly by scheme 1, while scheme 2 was the chief mechanism in liver suspensions.



Scheme 1



Scheme 2

These schemes are overall mechanisms and do not necessarily represent any fundamental difference between the individual reactions in the two tissues. Both are in fact consistent with ADP being the specific primary phosphate acceptor of the oxidative phosphorylation reaction. In the presence of labelled inorganic phosphate (P*), the following reactions are possible (APPP = ATP, APP = ADP, AP = AMP):

APPP → APP + P (*ATP-ase*)
APP + P* → APPP* (*oxidative phosphorylation*)
APPP* → APP + P* (*ATP-ase*)
2APP → APPP + AP
APPP* + AP → APP* + APP } (*myokinase*)
APP* + P* → APP*P* (*oxidative phosphorylation*)

Thus, if ATP-ase is much more active than myokinase, the ATP will be labelled mainly in the terminal phosphate group, which was what Krebs *et al.* (1951) found with muscle suspensions. If, on the other hand, the myokinase is relatively the more active, the two terminal groups of ATP would be equally labelled, as was the case with liver mitochondria in the experiments of Krebs *et al.* (1951).

The specificity for ADP of the oxidative phosphorylation reactions associated with the oxidation of α-ketoglutarate to succinate eliminates the possible participation of reaction (5). This is in agreement with Kaufman's (1952) studies of the soluble enzyme system which couples the reduction of DPN by α-ketoglutarate with phosphorylation. It is possible that coenzyme A monophosphate is an intermediate (Kaufman, 1952).

Nature of endogenous adenine nucleotide ~P

The presence of adenine nucleotide ~P in the sarcosomes is clearly indicated by the formation of HMP when adenine nucleotide is omitted from the normal reaction mixture containing hexokinase and glucose. It was confirmed by direct analysis of the sarcosomes. Since there is a substantial rate of oxidation in the absence of hexokinase and glucose, ADP must be continually supplied to the system (cf. Lardy & Wellman, 1952). It follows then that ATP formed during phosphorylation in the absence of added adenine nucleotide must be broken down again to ADP, i.e. that it must be readily accessible to ATP-ase in the preparation. This rate of breakdown is, however, the rate-limiting step in the rate of oxidation, since the addition of hexokinase and glucose increases the rate of oxidation. This finding and the formation of HMP under such conditions shows too that the ATP formed is also readily accessible to added hexokinase and glucose. The explanation given above for the phosphorylation of AMP implies that the ATP formed from endogenous adenine nucleotide must also be accessible to myokinase.

Since ATP formed from endogenous adenine nucleotide is readily accessible to ATP-ase, it follows that the preparations cannot contain any appreciable amount of ATP. The endogenous energy-rich adenine nucleotide must then be ADP. The

amounts of ADP found by direct analysis are quite high, namely 0·02 μmole/mg. sarcosomal protein or 1 molecule for every molecule of protein of molecular weight 50 000. Since so many individual enzymes have been located in the mitochondria and sarcosomes, it seems unlikely that the enzymes which specifically bind and react with ADP can comprise more than a few per cent of the total protein. Only a small fraction of the endogenous ADP is likely to be firmly bound to these enzymes. The remainder may be bound non-specifically to other sites, or may actually be concentrated in the intrasarcosomal fluid.

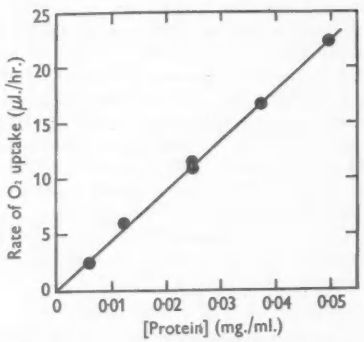


Fig. 13. Rate of oxidation of succinate (0·032 M) by different concentrations of Keilin and Hartree heart-muscle preparation. Phosphate buffer, pH 7·4, 0·07 M; ethylenediaminetetraacetate, 0·001 M. Temperature, 25°.

It was of interest to determine whether the endogenous ADP was bound more firmly than added ADP and was catalytically more active, as has been found to be the case with cytochrome *c* (Slater, 1950*b*; Tsou, 1952). The experiments described above showed, however, that unlike cytochrome *c*, ADP dissociated from the enzyme at high dilution and that the catalytic activity of the added ADP was of the same order of magnitude as that of the endogenous ADP. In contrast, Fig. 13 shows that the rate of oxygen uptake with succinate as substrate was exactly proportional to the concentration of the Keilin & Hartree (1947) preparation between 0·006 mg. protein/ml. and 0·05 mg. protein/ml. in the absence of added cytochrome *c* (ethylenediaminetetraacetic acid was used as the activator of the succinic oxidase system; see Bonner, 1953). If it is assumed that the concentration of cytochrome *c* is of the same order of magnitude as that of the site with which it combines on the particle and that a 10% departure from linearity at the lowest concentration would have been detected, and using the concentration of cytochrome *c* in the Keilin & Hartree preparation previously determined (Slater, 1949), it can be calculated that the K_m of bound cytochrome *c* cannot exceed 4×10^{-11} M. The K_m of added cytochrome *c* is about $1{\cdot}5 \times 10^{-5}$ M (e.g. see Fig. 3 of Tsou, 1952).

The K_m is not necessarily identical with the thermodynamic dissociation constant of the enzyme-substrate or enzyme-coenzyme compound (Briggs & Haldane, 1925; see Slater & Bonner, 1952), but it sets an upper limit for the dissociation constant. Thus, cytochrome *c* is extremely firmly bound to the particles of the Keilin & Hartree heart-muscle preparation, at least in the presence of ethylenediaminetetraacetic acid. Although the affinity of ADP for sarcosomes cannot compare with that of cytochrome *c* for the Keilin & Hartree preparation, it is still veryhigh (K_m of about 7×10^{-7} M) and much higher than would be suggested by the amounts of adenine nucleotide usually added in studies of oxidative phosphorylation. The requirement for the larger amounts is probably due to the instability of ADP in most types of enzyme preparations used in such studies.

The lower P:O ratio obtained in the absence of added ADP might indicate that, in the absence of an optimal concentration of ADP, some of the hydrogen-transferring steps involved in the α-ketoglutaric oxidase system proceed without phosphorylation.

Kinetics of oxidative phosphorylation

The experiments described on pp. 533–4 show that the oxidative phosphorylation reaction proper does not lag behind the hydrogen transfer. The lag of HMP synthesis behind hydrogen transfer, previously reported (Slater, 1950*a*) is due to the building up of a steady-state concentration of ATP. Kielley & Kielley (1951) have compared a lag of phosphorylation which they found after incubation of liver mitochondria with the one found by Slater (1950*a*). Since Kielley & Kielley (1951) followed phosphorylation by measuring disappearance of inorganic P, the accumulation of ATP would not cause any time lag. Kielley and Kielley's lag is, therefore, not the same as the one reported by Slater (1950*a*).

The perfectly linear kinetics obtained even during the first minute with a very low concentration of sarcosomes (Fig. 3) makes it unlikely that an intermediate of the type of succinyl phosphate is involved in oxidative phosphorylation. It is more probable that the concentration of any phosphorylated intermediate is of the same order of magnitude as that of the sarcosomal enzymes or coenzymes. This conclusion is in agreement with Kaufman's (1952) studies of his soluble enzyme system, which eliminate succinyl phosphate as an intermediate.

Measurement of yield of oxidative phosphorylation

The shape of the curves in Fig. 6 clearly show that, provided at least 35 units of hexokinase/mg. sarcosomal protein are used, the maximum P:O ratio is measured. The flat portion of the curve indicates that the hexokinase is reacting with all the ATP, none of which reacts with ATP-ase. As a routine, we use at least 100 units of hexokinase/mg. sarcosomal protein. Under such conditions, underestimation of the yield of oxidative phosphorylation due to the presence of ATP-ase can be neglected (cf. Slater, 1953*b*).

A possible objection to this conclusion is that ATP formed in the sarcosome might react more readily with ATP-ase in the sarcosome than with hexokinase in solution. We reject this interpretation for the following reasons. (1) The curves in Fig. 6 clearly reveal a competition phenomenon between ATP-ase and hexokinase. The interpretation just mentioned would not give curves of this type. (2) Reasons have already been given for believing that ATP formed even from the endogenous ADP of the sarcosomes is relatively free and capable of reacting with the soluble proteins myokinase and hexokinase as well as with the ATP-ase in the sarcosomes.

SUMMARY

1. Both oxidation and oxidative phosphorylation proceed at a uniform rate without any detectable time lag from the moment when heart-muscle sarcosomes are added to a suitable reaction mixture containing α-ketoglutarate.

2. Unless a very high concentration of hexokinase is used, there will be a slight lag in the synthesis of hexose monophosphate, due to the accumulation of adenosine triphosphate.

3. The maximum yield of oxidative phosphorylation (P:O) was obtained with rat-heart sarcosomes isolated in saline when 35 units or more of hexokinase/mg. sarcosomal protein were used.

4. Adenosine monophosphate (AMP) was phosphorylated at less than 1% of the rate of adenosine diphosphate (ADP). This slow phosphorylation of AMP probably requires first the formation of ATP from ADP present either as traces in the AMP preparation or in the sarcosomes and secondly sufficient myokinase to promote the phosphorylation of AMP. The much greater rate of phosphorylation of AMP by liver mitochondria found by other workers is very likely due to their higher content of myokinase.

5. The α-ketoglutaric oxidase system and the accompanying phosphorylation have a high affinity for ADP (Michaelis constant about 7×10^{-7} M).

6. Freshly prepared sarcosomes contain 0·02 μ-mole energy-rich phosphate/mg. sarcosomal protein (0·04 μmole total adenosine). This is probably ADP which is present in amounts sufficient to give nearly optimal rates of oxidation in the absence of added adenine nucleotide when a relatively high concentration of sarcosomes (1·25 mg. sarcosomal protein/ml.) is used. Experiments at greater dilutions gave evidence of dissociation of ADP from the α-ketoglutaric oxidase system.

7. The affinity of this endogenous ADP for the α-ketoglutaric oxidase system is of the same order of magnitude as that of added ADP. This is in striking contrast to the behaviour of endogenous and added cytochrome *c* in similar systems. From the proportionality of the rate of oxidation of succinate to the concentration of heart-muscle preparation in the absence of added cytochrome *c* it was calculated that the K_m of bound cytochrome cannot exceed 4×10^{-11} M.

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